STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC

VOLUME 19

1944

Published by
BIOTECH PUBLICATIONS
GENEVA, N. Y., U. S. A.

Printed in the U. S. A.

No. 4, October 1944

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STAIN TECHNOLOGY

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PARAFFIN SECTION THICKNESS— A DIRECT METHOD OF MEASUREMENT

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ABSTRACT.—Paraffin section thickness may be directly measured by re-embedding the sections under consideration, cutting them again at right angles to the original plane of sectioning, and taking direct measurements with a filar micrometer after staining and mounting. Conditions and materials with which relatively undistorted 3 and 5 μ sections were secured include (a) a hand-honed knife with a 23° facet bevel, set at a clearance angle of 7°, and (b) a hard paraffin (56–58°) embedding medium, preferably with 5% beeswax and 5% bayberry wax added. By taking direct measurements, it was found that bull testis tissue cut at a microtome setting of 10 μ averaged 10.82 μ in thickness. Settings of 5 μ and 3 μ resulted in sections averaging 5.25 and 3.31 μ in thickness respectively. Stages in sporogenesis of *Onoclea sensibilis*, Lewitsky fixed, were examined after sectioning at settings of 10, 5, and 3 μ to determine necessity for thin sections. For this material, it was indicated that mitochondrial preparations as thick as 10 μ were worthless, regardless of good fixation and proper staining. Three-micron sections give the best results.

INTRODUCTION

The question of the relation of actual thickness of paraffin sections to the microtome setting has been approached in a number of different ways. One familiar method is to determine the depth of focus of the section by means of the calibrated fine adjustment of the microscope. John (1929a) found this to be unreliable, and showed that in some cases the error might exceed the thickness of the section itself. Pusey (1939) describes the method of marking off 1 cm. on the paraffin block and then dividing 10,000 by the number of sections cut from this length, the resulting figure being the average thickness in micra of the sections cut at a given setting. This method might be satisfactory in cases where tissue compression is negligible, but it does not take into consideration the excess thick-

ness which might be met in extremely thin sections. John (1929b) measured section thickness using an optimeter, and Richards (1942) applied the principles of interferometry to section thickness measurements. The latter method requires the use of a special slide and cover glass, and illumination by sodium light. Both these methods involve equipment not available to the average laboratory.

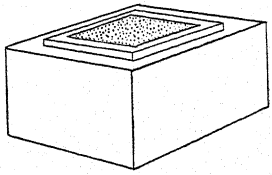
Dempster (1943) computed section thickness on the basis of percentage compression indicated by the ratio of floated-out section length to original block measurement. Using a 27° bevel angle knife at a 1° clearance angle, the thickness of sections cut at a setting of 10 μ, showing a compression of 27.5%, was computed to be 13.8 μ. With smaller microtome settings, increasing compression and excess thickness were found. At 5 μ setting, the compression was 57.8%, and the computed thickness 11.9μ, and at 2.5 μ setting, compression was 83.9% and the computed thickness 15.5 μ. The embedding medium was 52° "Bioloid" paraffin.

Having been at work for some time on a problem in plant cytology (i.e. the study of mitochondria and plastid primordia) requiring sections of 3 and 4 μ in thickness, it was surprising to the author to learn that on the basis of the last mentioned paper, laboriously prepared 3 μ sections might be anywhere between 11.9 and 15.5 μ in thickness. The literature concerning cytoplasmic inclusions contains numerous references to 4, 3, and even 2 μ sections. The figures of Senjaninova (1927) and Lewitsky (1925) show no distortion of cells, in spite of the fact that sections were cut 5 μ and below. The standard texts in microtechnic (Guyer 1917, Johansen 1940, Lee 1937, Mallory 1938, McClung 1937) all recommend sections 5 μ or below for mitochondrial preparations, and it was on the basis of these, and related papers, that 4 and 3 μ sections were used.

In view of the conflicting material in regard to distortion and excess thickness, a method was devised to determine directly the actual thickness of sections cut at 10, 5, and 3 μ settings, under conditions of sectioning identical to those of the cytological study mentioned above. Also considered was the problem of the necessity of cutting sections as thin as 3 and 4 μ for the study of the cytoplasmic inclusions under consideration. The relative tissue distortion appearing in sections cut at 10, 5, and 3 μ settings was also investigated.

Materials and Methods—General

The embedding medium consisted of 90 parts by weight of 56–58° "Bioloid" paraffin, 5 parts Eimer and Amend bayberry wax and 5



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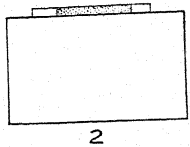

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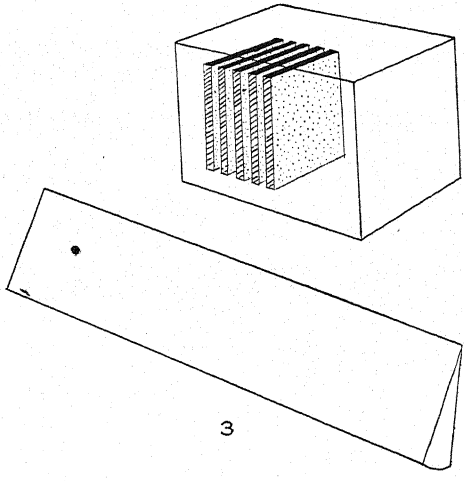

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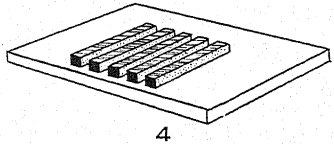

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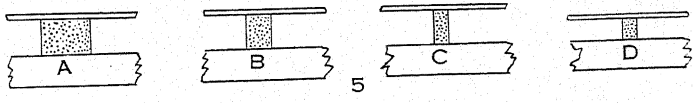

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Figure 1.—Diagram illustrating method of re-embedding tissue sections for resectioning. The paraffin section is placed on the surface of a trimmed paraffin block. When the heated tip of a small screw driver is brought over the section, the fusion of the paraffin of the section and that of the block embeds the tissue.

Figure 2.—Diagram of side view of figure 1.

Figure 3.—Diagram illustrating orientation for cutting of the re-embedded tissue sections.

Figure 4.—Diagram of a section cut from the block in figure 3.

Figure 5.— Diagrams illustrating slide, tissue and coverglass relations of the finished slides. (A) Original 10 μ section resectioned at 7 μ; (B) original 5 μ section resectioned at 7 μ; (C) original 3 μ section resectioned at 7 μ; (D) original 3 μ section resectioned at 4 μ.

parts Eimer and Amend yellow beeswax (Rugh, 1941). A Spencer rotary microtome, No. 820, was used. The knife had a 23° facet bevel, and had been hand-honed on a Droescher blue-green hone and finished on a Belgian yellow hone. It was set at a clearance angle of 7°.

Bouin-fixed bull testis tissue was used in the thickness determination and was also examined for cellular distortion at low microtome settings. Fertile frond segments of *Onoclea sensibilis*, Lewitsky-fixed, were used to determine the necessity of sections below 5 μ for accurate study of cytoplasmic inclusions. All the material was stained by the iron-hematoxylin method.

Determination of Section Thickness

To prepare sections for thickness measurements, they were cut at the desired microtome setting, re-embedded, then cut again so that after mounting and staining, a series of cross sections (Fig. 4) of the original sections would be ready for direct measurement with a filar micrometer.

Ribbons were cut from the same block of testis material at settings of 10, 5, and 3 μ. Re-embedding of the individual sections for re-sectioning was accomplished as follows: A block of the embedding medium was trimmed to a size slightly larger on its top surface than the sections of the ribbon being considered. A section from the ribbon was then cut off, and carefully centered on the top of the trimmed block (Fig. 1, 2). The tip of a small screw driver was heated and carefully brought to a point over the section. As this was slowly brought nearer the transferred section, the paraffin of the section and that of the block surface melted. When the heat was removed, the solidification of the medium embedded the tissue section in the top of the block. The procedure was repeated until a compact stack of sections was embedded. When sufficient sections had been transferred, a layer of the medium was added on top of them, and the block was then ready for trimming.

The block was oriented for cutting so that there would be no possibility of compression in the direction of section thickness measurement (Fig. 3). The sections were then cut and mounted in the usual manner. Following removal of the paraffin prior to staining, the slides were coated with thin celloidin by dipping them in 0.5%–0.25% celloidin in equal parts of absolute alcohol and ether. Hardening the celloidin was accomplished by partially air-drying the slides prior to placing them in 95% alcohol. This procedure prevents displacement or washing-off of tissue sections.

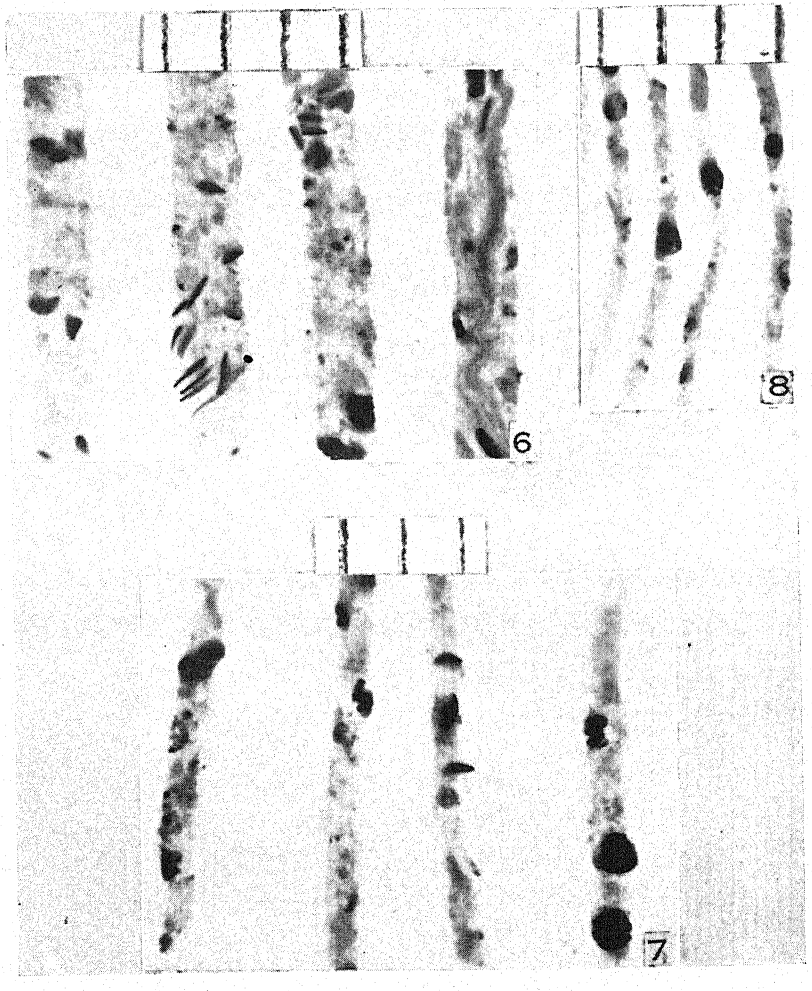


Figure 6.—Cross sections of 10 μ bull testis sections.
Figure 7.—Cross sections of 5 μ bull testis sections.
Figure 8.—Cross sections of 3 μ bull testis sections.
All figures 850×, Bouin fixation, iron hematoxylin. The stage micrometer spaces above each group of sections are equivalent to 10 μ each, and were photographed at same magnification.

For the sections cut at 10 and 5 μ settings, a second cutting at 7 μ proved satisfactory, the strips on the slide presumably being 10 μ wide by 7 μ high in the first case, and 5 μ wide by 7 μ high in the second (Fig. 5a, b). With the 3 μ sections, however, the 3 μ by 7 μ strips (Fig. 5c) did not all remain upright, and by the time the slides were stained and the cover glasses mounted, the majority of them had fallen over so that the 3 μ edge was no longer uppermost. Changing the second cutting settings of the 3 μ sections to 4 μ resulted in more satisfactory slides, and measurements were readily made on these, the strips 4 μ high by 3 μ wide remaining upright (Fig. 5d).

Table 1 summarizes the results of 30 determinations of section thickness at each of the original microtome settings. The measurements were taken with a Bausch and Lomb filar micrometer calibrated by means of a stage micrometer to use with a 44× objective. One dial interval was determined to be equivalent to 0.211 μ.

TABLE 1. THE RELATION OF AVERAGE THICKNESS OF SECTIONS TO MICROTOME SETTING.

Microtome Setting	Average No. Dial Intervals	Average Thickness	Average Excess Thickness
10 μ	51.3	10.82 μ	0.82 μ
5	24.9	5.25	0.25
3	16.53	3.31	0.31

The photomicrographs (Fig. 6, 7, 8) show characteristic cross sections of the original sections cut at the three settings used. Ten-micron spaces of a stage micrometer photographed at the same magnification are shown above each group of sections.

EXPLANATION OF PLATE 3

Figure 9.—Portion of a semeniferous tubule of bull testis. Bouin fixation, iron hematoxylin. Thickness 3 μ.

Figure 10.—Same as figure 9. Thickness 5 μ.

Figure 11.—Same as figure 9. Thickness 10 μ.

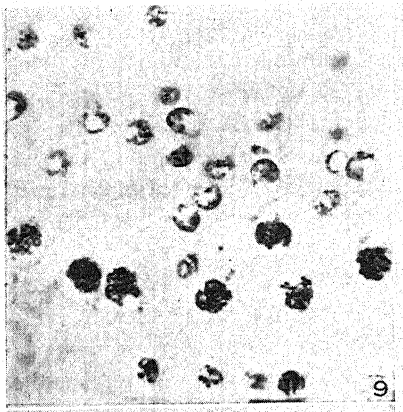
Figures 9, 10, and 11 are from the same block of tissue, and from the same slide. 500×.

Figure 12.—Young sporangium of *Onoclea sensibilis* showing stained inclusions of central sporogenous cell and tapetum. Lewitsky fixation, iron hematoxylin. Thickness 3 μ.

Figure 13.—Same as figure 12. Thickness 5 μ.

Figure 14.—Same as figure 12. Thickness 10 μ. Note poor differentiation of cytoplasmic inclusions which show clearly in figures 12 and 13.

Figures 12, 13, and 14 are from the same block of tissue and from the same slide. 850×.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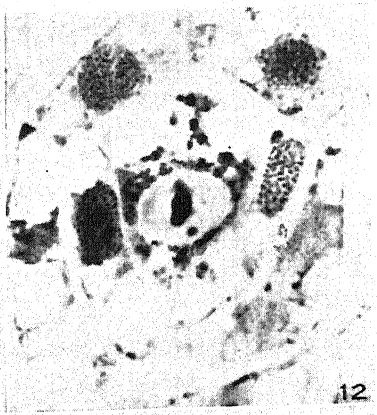
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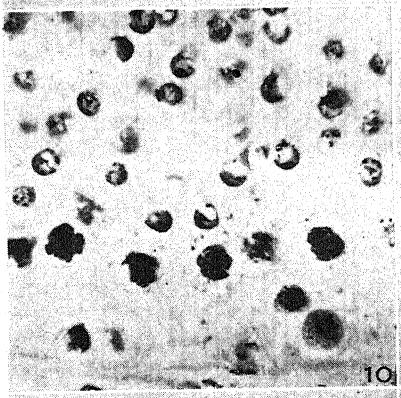
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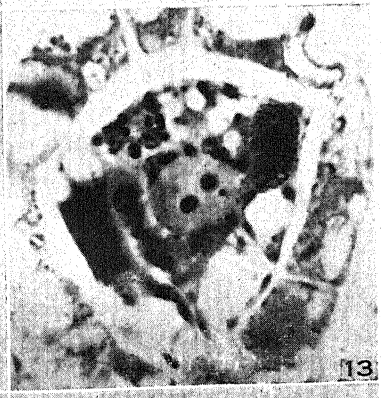
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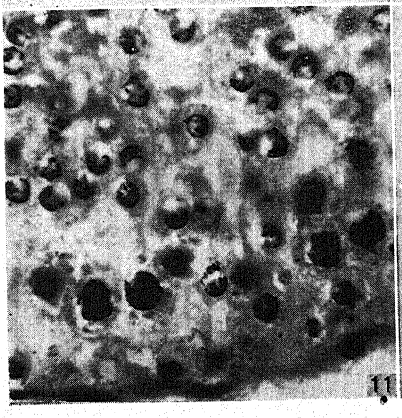
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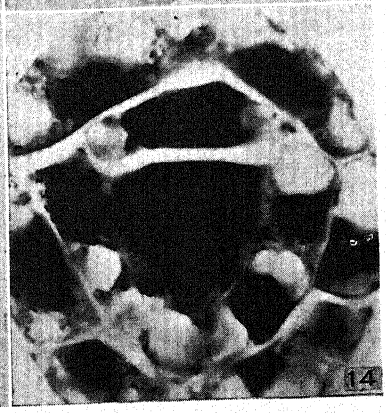
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The Necessity for Thin Sections

A block containing a segment of a fertile frond of *Onoclea sensibilis*, fixed by the Lewitsky method, was sectioned at 10, 5, and 3 μ. On each slide, a strip of 10 sections of each thickness of ribbon was mounted. The different thicknesses of tissue on a given slide were thus subjected to identical staining and differentiation times, and an accurate comparison of the three thicknesses made. Microscopic examination indicated that the 3 μ sections (Fig. 12) showed the clearest differentiation of the stained cytoplasmic inclusions. At 5 μ, (Fig. 13) these were still distinguishable, but not as clearly so as in the 3 μ sections. The 10 μ sections (Fig. 14) proved worthless for the study of the inclusions preserved by this method of fixation. All three photomicrographs (Fig. 12, 13, 14) are from the same slide. Identical exposure times were used.

Tissue Distortion

Bouin-fixed bull testis tissue was cut at microtome settings of 10, 5, and 3 μ. The different thicknesses of ribbon were cut from the same block, which had been trimmed so that its thickness was uniform throughout its length. On each slide were mounted 3 strips of 10 sections from each thickness of ribbon. Sections cut at all three settings were thus floated out, affixed, and stained simultaneously. Measurements on the finished slides showed the total length of the 90-section ribbon cut at 10 μ to be 403 mm. The 90-section ribbon cut at 5 μ was 372.5 mm., and that cut at 3 μ was 360.5 mm. The 5 μ ribbon was 7.5% shorter than the 10 μ one, and the 3 μ ribbon was 10.5% shorter than the 10 μ one.

The small amounts of compression shown by the measurements of total ribbon length do not appear to be significant when the tissue is examined microscopically. The nuclei of parallel stages in spermatogenesis shown (Fig. 9, 10, 11) appear to be of the same size in spite of the difference in the microtome settings at which they were cut. All three photomicrographs are from the same slide.

Discussion

The results indicate that under the sectioning conditions followed, relatively undistorted paraffin sections are possible with settings as low as 3 μ. While the nature of the tissue and the embedding medium undoubtedly have some bearing on the amount of distortion, it is believed that the acuteness of the bevel angle of the knife is the critical factor in securing undistorted thin sections. A closely graded series of knives with various bevel angles was not available and no extensive work was done to check this belief. However,

an earlier attempt to use a factory machine-honed knife with a 32° bevel resulted in hopelessly mangled and distorted tissue whenever the microtome setting was lower than 8 μ. Rehoning this knife at the same bevel on blue-green and then Belgian yellow hones produced no improvement in its cutting quality. Using the regular carrier on the Spencer 820 rotary microtome, the maximum clearance angle obtainable with this knife was 2.5°. It is possible that the use of a special carrier permitting a greater tilt might make for better cutting. This problem also requires extensive investigation which was not possible at this time.

The use of hard (56–58°) paraffin has proved to be most satisfactory when thin sections are needed. Previous work when pure paraffin of this melting point was used, indicated that with the same knife and the same clearance angle, 5 and 3 μ sections were not appreciably distorted. The addition of beeswax and bayberry wax improves the medium considerably, and ribbons cut at 3 and 5 μ retain their firmness even in hot weather.

On the basis of direct measurements, it appears that the excess thickness resulting from paraffin compression is nowhere near the amount indicated by indirect computations based upon abnormally compressed sections. At the three settings checked (10, 5, 3 μ), the average excess thickness was in all cases under 1 μ. Under similar conditions of sectioning, with similar tissue, the sections should never be more than 1 μ thicker than the microtome setting, at least when cutting 3 μ or above.

The necessity for thin sections in mitochondrial preparations is clearly shown by the failure of these elements to be sharply delineated in 10 μ sections subjected to identical staining and differentiation as sections of 5 and 3 μ. Five-micron sections in some cases appear satisfactory, but the clear definition of these bodies in 3 μ sections is not equaled in cells cut thicker. The trouble involved in securing a hand-honed knife of 23° bevel repays well in the undistorted thin sections produced.

Summary

1. A satisfactory method of direct determination of paraffin section thickness has been described.

2. Conditions and materials with which relatively undistorted 3 and 5 μ sections with excess thickness under 1 μ were secured include (a) a hand-honed knife with a 23° facet bevel, set at a clearance angle of 7°, and (b) a hard paraffin (56–58°) embedding medium, preferably with 5% beeswax and 5% bayberry wax added.

3. For material of a similar nature to that used in this study, mitochondrial preparations of 10 μ in thickness are worthless, regardless of good fixation and proper staining. The 3 μ sections give best results.

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FLUORESCENCE MICROSCOPY APPLIED TO ENTOMOLOGY AND ALLIED FIELDS

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ABSTRACT.—Fluorescence is a phenomenon observable in many substances including a wide range of biological constituents. By use of ultraviolet illumination and the proper fluorescent dyes, when needed, many details of structure and physiological differentiation are made apparent which by illumination with visible light are obscure.

The fluorescence microscope is a valuable adjunct to the study of fluorescence in biological materials. This instrument is discussed from a practical standpoint. The simplifications in the instrument which do not impair its efficiency are indicated.

The use of fluorochromes is discussed and a list of the most important of these is given. Important technics with the fluorescence microscope, including intravital microscopy, fluorescent photomicrography, and microspectroscopy, are described.

INTRODUCTION

Fluorescence is a phenomenon observable in many substances which, when exposed to ultraviolet irradiation, absorb energy and emit this in the form of light of a longer wave length than the activating rays. This response was set forth as a physical law by Stokes. From statements accredited to Helmholtz (1874) it is to be expected that the image of a substance will be better differentiated if the microscopic object itself were to emit the illuminating rays. The fluorescence microscope fulfills this condition, and, in addition, makes it possible to estimate qualitatively the constituents of fluorescing materials and the physiological state of living systems. The importance of fluorescence in biological material was recognized by Kohler (1904b), and during the period which followed, the valuable technics of fluorescence microscopy have been developed.

The use of the fluorescence microscope has brought about many important discoveries in the fields of applied biology. A comprehensive listing of these up to 1939 can be found in the review paper of Ellinger (1940b).

Since 1939 papers have appeared describing many phases of

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research with fluorescence technics. The distribution of vitamin A in various tissues has been described by Popper (1941), and by Cornbleet and Popper (1941); and Popper and Greenberg (1941) have reported the differentiation of vitamins A and A_2 in the livers of fish by this technic.

Investigations with the use of fluorescein in intravital studies have been advanced by Evans and Singer (1941) with their studies of the function of the eye.

The use of fluorescence technics for the demonstration of tuberculosis bacteria has been described by Richards and Miller (1941). Patton and Metcalf (1943) have extended the work of Bock and Oesterlin (1939) to recommend the use of fluorescence for the detection of protozoan parasites in blood smears. Plate 1-c.

The use of the fluorescence microscope has also been described in the study of the metabolism of water-soluble vitamins in the American roach by Metcalf and Patton (1942) and by Metcalf (1943a). The same author (Metcalf, 1943b) has also described the fluorescence characteristics of a new biological pigment isolated from the gonads of fireflies.

Although phenomenal results have been reported from the application of fluorescence technics, the technical difficulties, both real and imaginary, which accompany such have limited wide acceptance. It is the purpose of this paper to point out simplifications in equipment and methods which can be made without reducing the quality of the results, and to enumerate and describe the auxiliary technics which can be used to supplement visual observations.

In prosecuting this study, the authors have taken great care to avoid recommendations which have not been thoroughly tested in our own laboratories. When simplifications of equipment and technics are indicated, explanation has been attempted on a sound theoretical basis.

Apparatus

The apparatus required for fluorescence microscopy is essentially the same as for exact microscopic work under the usual conditions. It consists of a source of ultraviolet radiations which should be mounted upon a simple optical bench to insure critical focusing, a system of filters to limit the wave-length range of the illuminating beam, a good monocular microscope, and an auxiliary filter to remove stray ultraviolet light which penetrates the microscope.

The Light Source. The principal innovation in the equipment is the source of light. In order to excite the emission of fluorescent light by the object according to Stoke's law, the exciting radiation

must be rich in rays of short wave length. The production of fluorescence in biological materials is excited effectively by rays between 350 mμ and 400 mμ. For this purpose the high pressure mercury vapor arc lamp appears to be the most satisfactory (Gage 1941). The G. E. types H-3 or H-4 have been used extensively. These can be installed in any good microscope-lamp housing. The type H-4 requires a special base. A ballast transformer is required for either lamp. These lamps have a life of 1000 hours, are readily obtainable, and can be purchased complete with housing for a sum not significantly greater than the cost of a good research model microscope lamp.

Other light sources which can be used for this type of work are described by Ellinger (1940b). These include the D. C. iron arc; the carbon arc; the magnesium arc; iron-salt-cored carbon arc; low pressure mercury vapor lamp; and for less critical work with strongly fluorescent substances, tungsten sources such as a 500-watt projection lamp (Graham 1942), a six-volt lamp burned at double rating or a photoflood lamp.

The lamp housing should have the same attributes for use with the fluorescence microscope as for ordinary work. It should be equipped with a secondary source such as a condenser; it should have a diaphragm; and it should be mounted on a rod or track so that its position relative to the microscope can be accurately adjusted. The latter point is important.

Filters. In order to observe the fluorescent light produced by the ultraviolet beam, all visible light must be removed by filters. The most suitable for fluorescence microscopy with biological objects is No. 584 (new No. 5840) red ultra which is obtainable from the Corning Glass Works. This filter transmits radiations between 300 mμ and 400 mμ, and a very small percentage of visible light. Filter No. 586 (5860), violet ultra, transmits almost no visible light, but has an ultraviolet transmission only about one half that of number 584. It is useful where an absolutely black background is desired without the use of dark-field. Ultraviolet transmitting filters numbers 587 (5874); 597 (5970), the red purple ultras: and number 585 (5850), blue purple ultra, transmit too much visible light to be desirable for many phases of fluorescence microscopy. This is also true of number 986, red-purple Corex A, which is recommended by Gage (1941) for ultraviolet microscopy. This filter has a very high ultraviolet transmission down to 250 mμ but passes so much visible light as to be undesirable for most biological work.

The earlier investigators have made use of glass or quartz absorption cells 1 or 2 cm. in thickness and containing a 1% solution of *p*-nitrosodimethylaniline to remove the blue, green, yellow, and orange rays. Blue "Uviol" glass filters may also be used in addition to this absorption cell to absorb blue and violet light not trapped by the *p*-nitrosodimethylaniline. This combination allows the passage of light from 280 mμ to 400 mμ.

With the mercury arc, a small amount of visible red light passes the red ultra filter number 584 and must be absorbed. This can be done by using a glass cell one centimeter in thickness containing a 5% to 10% solution of $CuSO_4$ to which a few drops of H_2SO_4 have been added. The solution should be renewed at intervals. A blue green Corning filter number 428 (4308), can be used in place of the $CuSO_4$ solution, but it is not as effective in removing the heat rays; and its concentration cannot be adjusted to give complete absorption of the red light. This is very important where red fluorescing substances are to be studied.

If a piece of opal glass of the same size as the ultraviolet filter is provided, a change from fluorescence observation to visible light is almost instantaneous.

Almost any good microscope can be used with a mercury lamp for fluorescence observations. Certain reservations do apply however, for the most satisfactory results.

Mirror. The usual plane microscope mirror is satisfactory for fluorescence work. The use of quartz prisms or the relatively inexpensive evaporated aluminum-front surface mirrors, or the aluminum disks supplied commercially is desirable but unnecessary, providing the intensity of the ultraviolet beam is sufficiently great. Where the intensity is low, the use of these mirrors is essential.

Substage Condenser. It is commonly stated that quartz lens systems in the condenser on the source and the substage condenser of the microscope are essential for fluorescence microscopy. (Gage, 1941, Ellinger, 1940b, and others). This equipment is expensive, and, as our investigations have shown, is unnecessary for the production of fluorescence in biological materials. This fluorescence is excited principally by rays with wave lengths between 350 mμ and 400 mμ, and in this region glass is sufficiently transparent to allow a high percentage of the activating rays to penetrate.

In selecting optical equipment it is important to use non-fluorescing lens systems for all parts.

Three types of condensers are commonly used in bright field microscopy. Of these, the aplanatic has given the most satisfactory

results under the conditions necessary for fluorescence. This condenser consists of three lenses one of which is aspheric. It provides for a greater concentration of light than the Abbé and gives a greater spherical correction. Usually the aplanatic condensers are separable to provide for use with lower power objectives. The Abbé condenser usually found on student and medical model microscopes is entirely satisfactory for fluorescence microscopy. There is no serious loss in intensity with this type of condenser over the aplanatic. Achromatic condensers may also be used, but due to their construction with 5 or 6 lenses, there is a loss of intensity brought about by the light absorption.

The condenser should be fitted with a removable dark field disk. Both visual and photographic observations are made with this disk in place. This gives a luminous object against a perfectly black background with most of the ultraviolet rays escaping the microscope objective. For visual work where great brilliance is required, especially with immersion lenses and very high magnifications, the darkfield disk may be removed.

A cardiod darkfield condenser was tried with the fluorescence miscroscope. Although the results with the ultraviolet illumination were good, they were not sufficiently superior to compensate for the inherent optical limitations of this type of condenser.

When objectives of 20× magnifications or higher are used, a drop of petrolatum or water should be placed upon the condenser to serve as a connecting fluid between condenser and slide. This is essential for high intensity illumination. With low power objectives, it is necessary to remove the top lens or lenses of the condenser in order to illuminate the field.

Slides. Ordinary glass microscope slides from 1.2 to 1.5 mm. in thickness may be used. It is important that the thickness be no greater than 1.5 mm. The quartz and Corex slides recommended by Ellinger (1940b) and Gage (1941) offer no special advantages when the intensity of ultraviolet light is sufficiently great.

Mounting Media. The usual mounting media contain brightly fluorescent components, and substitutes are necessary. For temporary mounts, petrolatum serves well. This also serves as a satisfactory immersion medium for use with oil immersion lenses. For permanent mounts, iso-butyl methacrylate (duPont) as recommended by O'Brian and Hance (1940) has proved superior to all other mounting media. It may be used with or without a cover glass, is water white, has a satisfactory refractive index, is readily soluble in xylene or toluene, and is very stable. It does not discolor with age.

Objectives. Achromatic, fluorite, or apochromatic objectives are satisfactory. For color correction, the apochromatic system should be superior to the achromatic system, but the additional lenses which comprise the apochromatic objectives reduce the intensity of the image to a noticeable extent. In general, the same rules apply to the use and selection of objectives for fluorescence work as for bright light microscopy.

Oculars. As with the objectives, the same rules apply in the selection of oculars as with ordinary microscopy. Either negative (Huygenian) oculars or positive (compensating) oculars may be used. High power oculars should be avoided. Best results are obtained with a 5× or 10× ocular.

Auxiliary filter. Stray ultraviolet light which penetrates the microscope can cause a disturbing fluorescence of the eye and will fog a photographic film unless an auxiliary filter is used. Several types of filters are available commercially; however, in our experience where color photography was practiced, a liquid filter consisting of a 5% solution of $NaNO_2$ contained in a plane-sided glass cell 5 to 10 mm. thick was the most satisfactory. This filter may be placed as a cap on the ocular providing the latter has a sufficiently high eyepoint, at the point of the diaphragm in the ocular, or at the point of the diaphragm in the tube of the microscope. The last placement is the most convenient.

Such filters as the Corning number 306 (3060) "Noviol" shade O, the Leitz filter number 8574a as recommended by Popper (1941), or the Zeiss Euphos filter are colored and appreciably alter the color of the image. These are optically superior to the liquid filter at high magnifications. A satisfactory yellow filter can be made by cementing yellow or amber cellophane between two circular cover glasses with isobutyl methacrylate. Gelatin filters can be used if the natural fluorescence of this material can be avoided.

Fluorescence Microscopy by Incident Light

Intravital fluorescence microscopy was introduced by Ellinger and Hirt (1930) and has been found useful for the study of spontaneously fluorescent materials in living organisms, and for the study of opaque tissues and organs.

Apparatus. The apparatus consists of an ultraviolet light source identical with that described previously, used in conjunction with a microscope equipped for incident illumination by a vertical illuminator. This may be one of two general types. An internal mirror or prism may be placed in the objective to direct the ultra-

violet light, which enters by a side opening, down through the lenses of the objective, which act as a condenser, to the object. There fluorescence is induced and the image may be seen through the microscope. In commercial types such as the Leitz "Ultrapak", the Zeiss "Epicondensor", and the Reichert "Epiluminator", the light is reflected by a centrally bored annular mirror or its equivalent

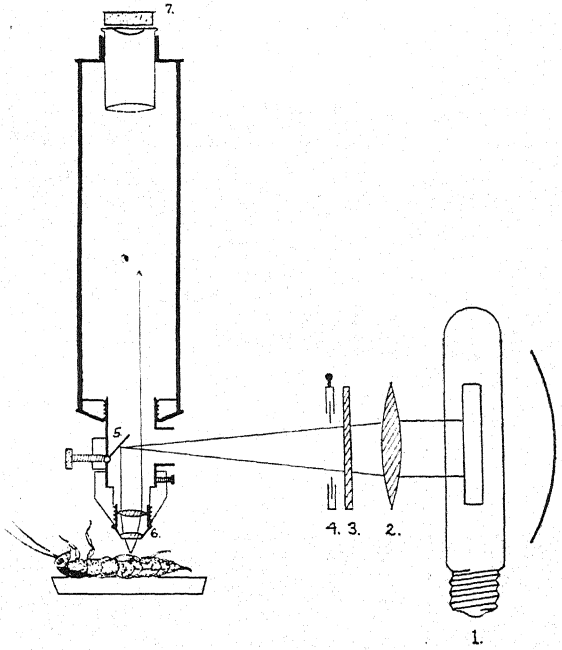


FIGURE 1. Equipment for Intravital Fluorescence Microscopy.

Key: 1. High pressure mercury vapor lamp
2. Condensor
3. Filter
4. Diaphragm
5. Front surface aluminum mirror
6. Divisible objective
7. Auxiliary filter.

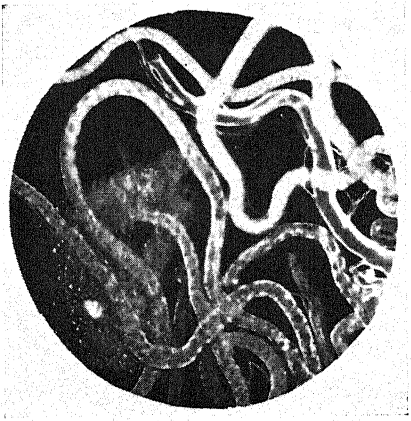
through an independent condensing system which surrounds the objective. These devices have been used very successfully in intravital studies but are relatively uncommon in this country.

A very simple vertical illuminator can be built which will give excellent results in intravital studies by fluorescence. Our apparatus (Fig. 1) consists of divisible objectives from which the upper portion with the diaphragm has been removed. These are threaded

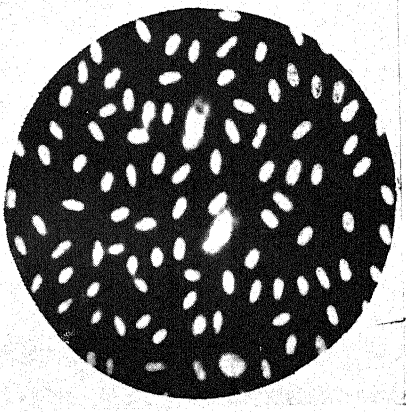
into a brass sleeve, which is threaded at its upper end with a standard objective screw. A circular opening drilled into the side of the sleeve allows the entrance of the ultraviolet light. Directly behind this opening is mounted a small front surfaced aluminum mirror which is fixed to a pivot which allows for focusing the light beam. The pivot is mounted upon a carriage controlled by a screw which effectively regulates the objective aperture, and serves as an internal objective diaphragm. Since in fluorescence microscopy the visible light source is in the object itself, there is no optical distortion due to oblique illumination. Although Ellinger (1940) states that the lenses of such an illuminator must be of quartz or of special ultraviolet transmitting glass, ordinary objectives may be used provided that the glass comprising the lenses or the cement used between the elements is non-fluorescent.

Only fine focusing is done in the conventional manner because of the fixed relationship necessary between the light source and vertical illuminator. The usual microscope stage is replaced by a stage capable of independent vertical adjustment, by which the object is brought into approximate focus. In use, the light source and vertical illuminator are aligned and the object is brought to the approximate focal point of the objective. The mirror of the illuminator is then tilted until the ultraviolet light beam is directed most intensely upon the object. This can be judged by the relative intensity of the fluorescence. By moving the mirror carriage the entire field can be evenly illuminated. When fluorescence is intense, a sharper image may be obtained by stopping down the diaphragm of the light source. With living material, continuous fine focusing is essential to give a three dimensional effect and to compensate for movements. Since a portion of the ultraviolet light is reflected back through the objective from the object, the use of an auxiliary filter is desirable. The $NaNO_2$ filter previously described is desirable for visual observations and essential for photography. Magnifications as high as 950× (with water immersion) have been used with excellent resolution. Plate 1-a. shows an intravital photomicrograph at 200× magnification.

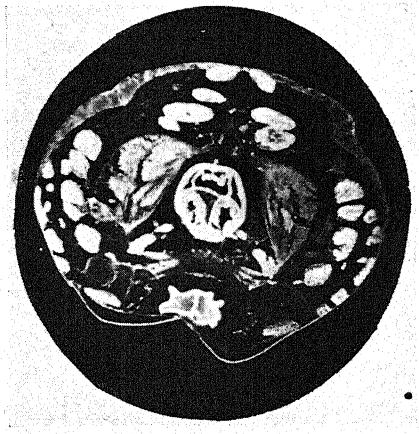
For intravital work it is frequently necessary to perfuse the tissues with a saline solution. Ordinary physiological salines are comprised of solutions of electrolyte salts which are non-fluorescent and can be used freely. Ellinger and Hirt (1930) have used water immersion lenses irrigated with a moving current of saline to keep the lens clear. Dry 16 mm. and 8 mm. lenses give good definition even through a film of saline.



A. Intravital photomicrograph of the malpighian tubes of *Periplaneta americana*. From top to bottom: free riboflavin in the lumen of the tubes; a section of trachea; cytoplasmic riboflavin; and fat body. Magnification 200 ×. $NaNO_2$ auxiliary filter. Agfa Superpan Supreme.



B. Fixed cross section of larva of *Chironomus* sp. Stained with acridine yellow. Multicolored effects are obtained with a single stain. Magnification 200×. $NaNO_2$ auxiliary filter. Agfa Superpan Supreme.



C. *Haemoproteus* parasites in an avian blood smear. Stained with berberine sulfate. Magnification 900 ×. wratten G filter. Agfa Ultraspeed pan film.

PLATE 1

Photomicrographs by fluorescence of fresh, and fixed tissues; and of parasites in avian blood cells.

Histology by Fluorescence

The application of fluorescence technics to studies of microscopic anatomy and physiology demonstrates the distinct possibilities of these methods. Fluorescence finds its greatest application in the differentiation of functional tissues within an organism. In this application, the tissues appear in various colors depending upon their physiological state. Such studies can be made intravitally, with frozen or fixed sections, and with or without the use of fluorescent dyes. Some tissues contain natural fluorescent pigments which illuminate the tissues brightly when excited by ultraviolet radiations. These tissues may be plainly traced, and in the case of intravital studies, the physiological state may be estimated without the intervention of any foreign material. Naturally fluorescent materials frequently encountered include several flavin compounds (such as riboflavin), carotenes (vitamin A), chlorophyll, porphyrins, certain of the alkaloids, etc.

If natural fluorescence is not present or does not produce enough intensity for the study at hand, dyes may be added. The use of fluorescent dyes is of value in intravital work as well as with fixed tissues.

Fluorescent Dyes. Fluorescent dyes or fluorochromes (Haitinger 1938) include various coal tar dyes, and plant extracts such as certain alkaloids and plant pigments. They are applied to the tissues in a manner comparable to the use of usual histological stains. The coal tar dyes used can be classified into five groups. These include: xanthenes, acridines, thiazols, amino-azines, and azo dyestuffs. Specific dyes from these groups are listed in table 1 along with their properties. The principal dyes for fluorescence staining are italicized.

A most remarkable feature of these materials is their intense fluorescence at great dilutions. Most of them give a strong fluorescence color at dilutions of 1×10^{-10} and in the case of fluorescein, at a dilution of 1×10^{-14}. At such dilutions the daylight color of the strongest dyes is imperceptible.

Staining Procedure. The preparation of tissues for fluorescence staining is not significantly different than for usual histological technics. The tissue is fixed, dehydrated, infiltrated, sectioned, placed on ordinary microscope slides with a very thin film of albumin, stained, and mounted. The only differences are in the fixatives selected, the stains used, and the final mounting medium.

The salts of heavy metals, with the exception of zinc, halogen radicals, and nitro compounds, exert a quenching effect upon fluores-

cence. For this reason, fixatives which contain these must be avoided. The recommended fixative for most fluorescence work is 4% to 10% formalin. Dietrich's fluid (also known as Kahle's), which consists of a mixture of 30 cc. of 95% alcohol, 10 cc. formalin, 4 cc. glacial acetic acid and 60 cc. distilled water, has some advantages over plain formalin and can be used without effecting fluorescence. Haitinger (1938) recommends a fixative of 7 parts of 3% K_2CrO_4 solution, 2 parts formalin, and one part acetic acid.

TABLE 1.—IMPORTANT FLUOROCHROMES

Chemical Group	Fluorochrome	Color Index	Source*	Fluorescent Color	Use
Acridines	*Acridine orange*	788	N.A.	Orange-red	General
	Acridine yellow	785	N.A.	Yellow-green	General
	Acriflavine	790	Merck	Yellow	General, trypanosomes
	Coriphosphine O	787	Grb.	Yellow	General
	Phosphine 3R	793	Grb.	Yellow	Fat
	Rivanol		Metz	Green-yellow	Protozoan parasites
Xanthenes	Eosin Y	768	N.A.	Yellow	
	Fluorescein	766	N.A.	Green-yellow	Vital stain
	Rhodamine B	749	N.A.	Orange to red	Fat stain
Thiazoles	*Primulin*	812	E.A.	Blue-violet	General, virus and bacteria
	Thioflavine T. G. Ext.	816	E.A.	Light blue-green	General, virus and bacteria
	Titan yellow	813	Grb.	Blue	Fat
Quinone-imines	Magdala red, echt	857	Grb.	Red-orange	Fat
Phenyl methanes	Auramine O	655	N.A.	Yellow	Acid fast bacteria
	Acid fuchsin	692	N.A.	Red	Counterstain.
Azo dyes	Congo red	370	N.A.	Dull red	Counterstain.
	Thiazine red	225	Grb.	Violet	Fat
Alkaloids	Berberine sulfate		Merck	Yellow	Protozoan parasites (malaria)

*N.A.—National Aniline & Chemical Co. Metz—H. A. Metz Laboratory
Merck—Merck & Co. E.A.—Eimer and Amend
Grb.—Dr. G. Grubler & Co.

Almost all of the dyes mentioned are applied to the tissues from water solutions with dilutions ranging from 1/1000 to 1/10,000. This is near the range of saturation for many of the materials. At such dilutions, the staining time is from ½ minute to 5 minutes. The stains may be applied either by dropping the stain directly onto the sections or by dipping the slide as in usual procedures. After staining, the sections are washed in distilled water, dehydrated

through an alcohol series, cleared in xylene, and mounted in isobutyl methacrylate for a permanent mount or in petrolatum for a temporary preparation. Haitinger (1938) recommends a mounting medium consisting of 10 g. of clear, whitish gum arabic dissolved in 10 cc. of water and 5 cc. of glycerol with 1 g. of chloral hydrate added.

In intravital work the functions of living organs can be effectively studied by injecting fluorescent dyes into the organ to be studied or into the animal's blood stream. For such work the dyes employed must be water soluble, non-poisonous in the useful dilutions, non-diffusable in the living body, and must exhibit intense fluorescence in great dilution. The two most suitable dyes are the acid dyes, fluorescein or sodium fluoresceinate (uranine), and the basic dye, acriflavine hydrochloride. These are used as 0.1% to 1% solutions in physiological saline. Fluorescein is chiefly of use in the study of the function of organs. The color of its fluorescence varies with pH and consequently it is of use as an intravital hydrogen ion indicator. These color changes are easily visible at dilutions of 1/10,000,000. In dilute solutions the intensity of the fluorescence has a definite relation to the concentration of the dye. It thus serves as an indicator of the amount present. The relation of color to pH is described by Ellinger (1940a); pH is the only cause of change in color of this dye.

Acriflavine hydrochloride is used to differentiate cell structure. It is less intensely fluorescent than fluorescein and its fluorescence is partially quenched in basic solution so that it appears most readily in organs with an acid reaction.

Such dyes as rhodamine B, primulin yellow, thioflavine, berberine sulfate, vitamins A and G, Rivanol (2-ethoxy,–6,9,-diamino acridine lactate) and atebrine can also be used as intravital fluorescent stains. Rhodamine B reacts in a manner intermediate between fluorescein and acriflavine. The vitamins A and G can be used well as fluorescent dyes especially in studies of their metabolism. Such materials as Rivanol and atebrine can be studied and their therapeutic action estimated by their fluorescence reactions.

Tissue Differentiation. This phase of fluorescence microscopy is of especial interest to histologists, morphologists, and physiologists. The greatest advantage of the method is that the differentiation of tissue elements is varicolored without counterstaining. See plate 1-b. The differences in color produced by one stain may be explained by several factors.

The acridine and thiazol dyes are selectively absorbed by tissues,

and the fluorescence color produced varies greatly with the tissue to which the dye is fixed. This color differentiation can be partially explained, in the case of the acridine dyes by the fact that the fluorescence color is effected by pH. Since these materials also show differential absorption by various tissues, the two factors combine to produce a great variety of fluorescent colors. Dyes are taken up by various tissues at greatly different rates, and the contrast obtained is greatest when one tissue element is allowed to absorb a maximum amount without permitting any to be absorbed by the surrounding materials. The final fluorescence color visible to the eye is addative with the color of the natural fluorescence of the tissue being stained and the fluorescent color of the fluorochrome. The shade obtained depends upon the respective intensities of the two components.

As has been pointed out, a great variety of materials act as fluorochromes. The number actually needed for satisfactory permanent histological preparations can be limited to fewer than six. The acridine dyes, especially acridine yellow and coriphosphine O are the best general purpose stains. With either of them, most tissue sections exhibit very pleasing color differentiation. The thiazol dyes, thioflavine and primulin yellow, do not give as great color contrast as the acridines but give more intense fluorescence and are especially valuable for studies of musculature and structure. These are general purpose dyes. Special dyes and dye combinations may be used for specific purposes.

Although the best application at the present time for these technics appears to be the differentiation of tissues and organs rather than intracellular structure, cell structure and nuclear structure can be shown by special technics. Acridine yellow, acridine orange, coriphosphine O, phosphine 3R, and acriflavine all differentiate nuclei from cytoplasm clearly. This is also true of berberine sulfate. By staining with acridine yellow or coriphosphine O, and counterstaining with Congo red or acid fuchsin, a very distinct color differentiation of cytoplasm, nucleus, nucleolus, and chromatin can be obtained. This stain is applied by first staining for two minutes with Congo red and then two minutes with acridine yellow. The color differentiation is extremely sharp. Similar results can be produced with coriphosphine and fuchsin. A 2-to-1 mixture of these is effective after several minutes of staining. All of these dyes used in 0.1% solutions will give complete staining in one or two minutes.

Differentiation of Muscle. Almost all fluorochromes stain muscle

fibers. These appear light blue with acridine yellow, green with acriflavine, bright scarlet with acid fuchsin, blue with neutral red, and bright blue with primulin yellow. The most striking demonstration of muscle is produced by the diazotization procedure of Haitinger (1938), using the thiazol dyes, especially primulin yellow. The tissue is stained as usual with this dye and immediately after is washed in water and placed in a 2% aqueous solution of $NaNO_2$ to which has been added an equal volume of 10% HCl. The material remains in the bath about 15 minutes and is washed and treated for 15 seconds to a minute with a phenolic compound such as bromophenol, tetrabromophenol, resorcinol, or α-naphthol. By this procedure a polyazo dyestuff is developed in the tissue similar to the diazo dyes found in textiles. The fluorescence color produced depends upon the developing agent. For example primulin yellow diazotized with α-naphthol produces an incandescent yellow-white color in muscle tissue. The bromophenols give bluish colors and resorcinol, green. This same procedure can be followed with thioflavine and titan yellow and serves with other types of tissue as well as with muscle.

Differentiation of Nerve. Acridine yellow gives excellent differentiation of neuropile and cortical nerve tissue. The former appears light blue while the latter is light yellow to orange. With acridine orange the neuropile is blue gray and the cortical tissue orange brown. With acriflavine the neuropile appears blue and the cortical tissue yellow brown. Diazotization of primulin yellow gives good detail. The cortical tissue becomes dark violet to brown and the neuropile buff. Haitinger (1938) recommends diazotized thioflavine for nerve tissue.

Differentiation of Fat. Fat tissue in the fresh state has a natural blue to blue-violet fluorescence which may be confused with other blue fluorescent tissues. The fat can be differentiated by a variety of fluorochromes. The color produced varies with fixed and unfixed tissue and with the length of time in the fixative. This effect is generally a matter of a change in the shade of the fluorescent color. Popper (1941) has studied the distribution of vitamin A in fatty tissues and says that this material can be considered a vital stain for fat. He used a 1% aqueous methylene blue solution to stain fats for fluorescence examination. The staining requires 10 seconds. This causes the fat to fluoresce blue without obscuring the brighter vitamin A fluorescence. It is observable in visible light. Phosphine 3R, 1% aqueous solution, is a good fat stain. Three-minutes exposure produces a silvery white color. Popper (1941) says that

this stain differentiates more fats than can be seen with usual stains. He also used thioflavine S, 1% solution, which gave a violet fluorescence to the fat against a yellow background. This dye stained fewer lipids than phosphine 3R. The same author found magdala red or rose Bengal, used in 0.1% solutions and exposed for one minute, satisfactory. Haitinger (1938) recommends a freshly prepared alcoholic solution of chlorophyll which gives a fiery red fluorescence to fat, but this is impermanent. Several of the acridine dyes may also be used satisfactorily as fat stains.

Fluorescence Photomicrography

The image produced by the fluorescence microscope appears very bright and distinct by reason of the great contrasts, vivid colors, and the dark background; however, the light intensity is very low. No measurement of the intensity can be obtained with the usual photoelectric exposure meters. Exposure of photographic materials can be determined by selecting the optimum from a series of negatives exposed with the time of each exposure doubled. For photomicrography the dark-field disk and the auxiliary filter are always necessary in order to protect the emulsion from fogging by stray ultraviolet light.

From our experience, the exposure times for black and white film (Weston 50) using a 35 mm. camera with both the camera lens and the eyepiece in place ranges from 2 seconds to 5 minutes. The basic exposure for this type of condition is about 10 seconds.

When the durability of the preparation is not influenced by continuous ultraviolet radiation, [such as studies of vitamin A (Popper and Elsasser, 1941)], excellent results can be obtained by using Kodachrome (daylight). The exposure may be determined by calculation from a series of exposures made on black and white film or by a series of exposures made upon the Kodachrome itself. The selection of the method depends upon the requirements of the individual. Although color film gives excellent records and good illustrative material, the difficulties in duplicating exposure conditions make the value of the transparencies doubtful for standards of comparison for pH etc. Exposures of Kodachrome vary from 1–15 minutes with an average exposure time of about 2 minutes. Due to the low intensities, magnifications in excess of 500× are impractical. The microscope and camera must be mounted on a solid stand upon a vibrationless floor due to the long exposures.

Discussions of fluorescence photomicrography are to be found in Franke (1935), and Ellinger (1940).

Fluorescence Microspectroscopy

Positive identification of fluorescent materials in tissues can be made with a microspectroscope. It is possible to make spectroscopic measurements on extremely small areas of histological sections and isolated tissue elements.

The spectroscope equipment necessary may be either of the direct vision type employing Amici prisms or a grating type such as the Jelley microspectroscope (Jelley, 1936). In either case, calibration is simple since the mercury lines are easily observed by removing the filters from the light source.

Most fluorescent substances show an emission spectrum with a well defined maximum and these can be compared with a sample of the pure material suspected present by photographing the spectrum and comparing the negative densities. Fluorescence spectra of a number of biological materials have been determined and are to be found in the literature (Dhere 1939).

Conclusion

In the foregoing paper it has been the purpose to set forth the technics of fluorescence microscopy as they apply to biological research. The apparatus described has been thoroughly tested for many types of investigations over a period of three years and has proved satisfactory.

Although the fluorescence microscope as a research instrument has already made important contributions to the knowledge of biological phenomena, its possibilities for biological research are still limitless.

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PICRO-MALLORY

AN EASILY CONTROLLED REGRESSIVE TRICHROMIC STAINING METHOD

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ABSTRACT.—Three modifications of Mallory's connective tissue stain are described and some features of the action of picric acid are discussed.

In the first and most critical method the nuclei are stained in an iron hematoxylin and then differentiated in a picric acid solution containing orange G. This not only differentiates the nuclei, but stains all other elements yellow. The section is then washed in running water to remove the yellow color from all tissues except those which are to remain yellow in the final preparation (usually the erythrocytes). The section is next stained in an acid fuchsin mixture and then differentiated until the desired depth and contrast is obtained. Staining in anilin blue follows and this in turn is differentiated to suit. The section is then dehydrated and mounted.

In the second method the nuclei are stained in hemalum (e.g. Harris's) for a short time; the section is then rinsed and immersed in a mixture of picric acid and acid fuchsin and thereafter is differentiated; it is next passed into anilin blue w. s. and then differentiated and mounted as before. This is less critical than method I, but can be applied to large batches of slides at a time.

The third method is a one-solution method. After staining the nuclei in hemalum, the section is immersed in the "Picro-Mallory" solution, differentiated briefly, dehydrated and mounted. This modification, while being the least critical, is most suitable for routine use when the tissues have been fixed in a fluid containing chromate; the other commonly used fixatives, while giving useful results, are not so good.

This paper describes three modifications of Mallory's trichromic stain evolved from a previously published method (Lendrum and McFarlane, 1940); the name "Picro-Mallory" has been used for all the evolutionary stages of the stain. The most critical technic

[1]Part of this work was done while holding a Beit Memorial Fellowship.

(Method I) is applicable after all the commonly used fixatives, and it is of an easily controlled regressive character allowing the individual worker to emphasize the element in which he is most interested by varying the staining times. Up to the present, Method I (p. 35) has proved of great use in demonstrating the cytological changes in the pituitary (McLetchie, 1943), in showing many of the special features of spleen histology and in the investigation of the microscopical changes in the kidneys in nephritis. Apart from such highly critical aspects of the stain it has been possible by modification of the various solutions to evolve two additional less critical methods applicable to large batches of sections at the same time, the results so obtained being very good. In all cases the tissue elements stain as in Mallory's connective tissue stain.

In Mallory's method the erythrocytes are yellow, the cytoplasmic elements red (with certain exceptions) and the connective tissue blue. This technic has proved uncertain in the hands of many workers and further suffers from the inherent drawbacks that it is not under control and it is applicable only after chromate-containing fixatives or after suitable mordanting. Many modifications have been published since the original technic was described; of these Masson's is probably the best known and gives constant results after most fixatives. This stain is largely progressive in nature and has a further disadvantage in that all the acidophilic elements are colored red. Lendrum and McFarlane (1940) were able to get the classical colorations of Mallory's method by treating the section, after nuclear staining, in an alcoholic solution of picric acid containing orange G, and then following Masson's technic. This modification, however, did not increase control over the density of the dyes nor did it enable emphasis to be placed on one element in preference to another to any great extent. Much work has been done to overcome these difficulties and as a result two solutions have been evolved, one of which differentiates the red elements in an easily controllable manner, while the other removes excess of anilin blue from the cytoplasmic elements.

In the search for a "red differentiator", many substances and various combinations of substances were tried out. Alcohol in varying concentrations was found to remove the fuchsin with a rapidity increasing as the concentration of the alcohol increased; this method is not suitable as it does not act very rapidly and the action is non-specific, i.e. it removes the red from all elements at about the same rate. Alcoholic solutions of phosphotungstic and phosphomolybdic acid showed a marked improvement over the

alcohol, as the red dye, in this instance, was removed first from the connective tissue and then from the cytoplasmic elements. . This action was not sufficiently strong on sections of tissue fixed in chromate solutions and further tended to remove the yellow color from the erythrocytes. Such solutions if used in Masson's technic in place of the aqueous solution of phosphomolybdic acid allow of much greater control. Alcoholic solutions of picric acid remove the red dye with a rapidity increasing with the concentrations of the two substances, and when saturated solutions of picric acid in absolute alcohol are used, the dye is removed very rapidly. Such a solution, while acting as a true differentiator, does so in the opposite manner from that desired, i.e. the red dye is removed first from the cytoplasmic elements and then from the connective tissue. In this way a Van Gieson effect is obtained. However, alcoholic solutions of picric acid and phosphotungstic acid were found to have the requisite action when the strengths of the various substances were properly adjusted. The strength of the differentiating action is dependant on the concentration of alcohol and of picric acid. Absolute alcohol containing 40 g. per 100 ml. phosphotungstic acid (i.e. approximately saturated) and 4 g. picric acid (PTA: PA = 1: 10) is the strongest differentiator. A ratio of about 10:1 is necessary if a Van Gieson effect is to be avoided. By diluting such a differentiator the rate of differentiation can be adjusted to suit the individual worker and the fixative in use.

Another disadvantage often encountered in the various Mallory modifications is that after staining in anilin blue the red elements have taken up some of this dye and have become degraded to a purple. In order to correct this a "blue differentiator" was evolved. It contains the same substances as the red differentiator in different proportions. It was found that alcohol in its lower concentrations removed the blue dye in a non-specific manner and at the same time removed the yellow color from the erythrocytes. The addition of phosphotungstic acid rendered the action specific; the addition of picric acid retained the color in the erythrocytes. This solution makes it possible to stain the connective tissue adequately and then to remove the blue dye which has overlayed the red of the cytoplasmic elements.

Before outlining the technic the action of picric acid will be discussed as it presents many interesting features of possible use to the specialist.

In Method I, after sections have been stained in the nuclear stain, they are immersed in saturated 80% alcoholic picric acid containing

0.25% of orange G. This differentiates the nuclei and at the same time stains all the elements yellow. When the section is washed in tap water, the dyes are washed out of the various elements at a rate depending on their affinity for the picric acid. If a section is observed at intervals throughout the process of washing, the yellow color will be seen first to leave the connective tissue, after which it comes out of the cytoplasm, although certain granular contents may hold it for a longer period. Fibrin resists decolorization for a still longer time, and erythrocytes are the last to be differentiated, being the most picrophilic. By controlling the washing time, it is thus possible to remove the picro-orange almost completely from all elements, except from the erythrocytes, these being left a robust yellow. If at this stage the section is passed into a solution of acid fuchsin, all elements with the exception of the yellow erythrocytes will rapidly take up the red dye. The amount of picric acid in the erythrocytes is sufficient to deviate the red from these cells. However, if the sections are left for a sufficient time in the red dye, the picric acid will be removed and the acid fuchsin will then color the blood corpuscles. The greater the amount of picric acid in elements other than the erythrocytes the longer these elements will resist staining with the red dye. Most frequently the deviating action of the picric acid is used to retain the yellow color in the erythrocytes and so to differentiate the red cells from such elements as fibrin; the specialist may, however, use the action in other ways by controlling either the differentiation of the picric acid during the washing or by carefully watching the section while in acid fuchsin, so that elements normally possessing similar tinctorial characteristics may be differentiated. In general it may be said that all tissues which are picrophilic have a similar affinity for acid fuchsin and further that acid fuchsin is deviated for a time from elements containing picric acid; this effect is directly proportional to their content of the acid.

During the investigation into the action of picric acid, Van Gieson's mixture was examined with the following interesting findings. If saturated aqueous picric acid is added, a few millilitres at a time, to a 1% solution of acid fuchsin, and sections are stained in the various solutions so obtained, the following sequence of events will be noted: When the proportion of picric acid to acid fuchsin has reached about 1:4 or 1:2 it will be seen that all the elements are a deep red with the exception of the erythrocytes, which are a brilliant yellow. As the proportion of picric acid increases, the cytoplasmic elements take the yellow, and the red dye does not stain so intensely in any

of the elements. As the proportions of the two substances approach those of the commonly used Van Gieson's modifications (picric acid to acid fuchsin 10:1), the picric acid gradually deviates the fuchsin from the acidophilic cytoplasmic structures and in the final Van Gieson only the connective tissue takes the red. Further increase in the picric acid content prevents the red dye from staining any of the elements. This principle is used in the routine "Picro-Mallory" method of staining large batches of slides when it is desired to avoid all unnecessary complications.

Solutions and Reagents

1. *Picro-orange.*

80% alcohol saturated with picric acid	100 ml.
Orange G. (C. I. 27)	0.25 g.

Sections can be left in this solution indefinitely without coming to harm.

2. *Acid fuchsin mixtures.*

Acid fuchsin (C. I. 692), Biebrich scarlet (C. I. 280) and ponceau 2R (C. I. 79) may be used as 1% solutions in 1% acetic acid. It has been found that a mixture of equal parts of 0.5% acid fuchsin and ponceau 2R gives a large variety of pleasant color tones suitable for use in method I.

Picro-acid-fuchsin (method II).

Picric acid	0.2 g.
Acid fuchsin	0.8 g.
2% acetic acid	100 ml.

3. *Phospho-picric differentiators.*

Stock solution

Phosphotungstic acid	25 g.
Picric acid	2.5 g.
95% alcohol	100 ml.

A. Red differentiators:

Red differentiator for the critical method (I)

Stock solution	40 ml.
95% alcohol	40 ml.
Water	20 ml.

This may prove too rapid in action for tissues fixed in some fixatives and for certain workers. Further dilution with water slowly decreases the rapidity of action and so the strength can be made to suit individual needs. In certain instances, e.g. when tissues have been left in chromate-containing solutions for a long time, decolorization is not sufficiently rapid; all that is necessary in these cases is to

transfer the section from the red differentiator into a saturated solution of picric acid in 80% alcohol, after which it is again rinsed in the red differentiator to re-mordant with phosphotungstic acid. Since the connective tissues will have been decolorized in the red differentiator to begin with, the action of the picric acid is to remove the red dye from the parenchyma as explained above (p. 31).

Red differentiator for the routine method

Stock solution	40 ml.
Water	60 ml.

This mixture is such that, in this department where tissues are fixed in Helly's fluid, differentiation is adequate in about five minutes. By increasing or decreasing the strength of the alcohol the action may be made more or less rapid as desired.

B. *Blue differentiators:*

Blue differentiator for the critical method (I)

Stock solution	20 ml.
Water	80 ml.

Blue differentiator for the routine methods (II & III)

Stock solution	10 ml.
Water	90 ml.

The strengths given will be found to be useful, but more rapid or slower action may be obtained by increasing or decreasing the concentrations respectively. The use of a blue differentiator for an adequate time cannot be over-emphasized. The resulting clarity and brilliance of the histological picture is largely dependant on the removal of all the excess anilin blue from the section.

4. *Anilin blue* (*C. I. 707*).

This solution is made up as advised by Masson, i.e.: add to 100 ml. boiling distilled water 2–3 g. anilin blue, boil for a few minutes and then add 2.5 ml. glacial acetic acid; allow the mixture to cool and then filter. Many different samples of anilin blue from different makers have been used from time to time and all have proved satisfactory. The acidity of this solution must be maintained and care must be taken not to carry over into the anilin blue any of the red differentiator.

5. *One solution Picro-Mallory.* (*Method III*)

Picric acid	0.2 g.
Acid fuchsin	1.0 g.
Anilin blue	2.0 g.
Phosphotungstic acid	1.0 g.
2% acetic acid	100.0 ml.

Preparation: To 100 ml. water add the dyes and phosphotungstic acid. Bring to a boil and allow to cool. Add 2 ml. glacial acetic acid and filter.

There are several points of importance with regard to this solution. The proportion of picric acid to acid fuchsin must be fairly constant. Similarly the amount of phosphotungstic acid present strongly influences the ability of the anilin blue to stain the connective tissues. The more acid present the more the blue is deviated from the tissues. The proportion of acid fuchsin to anilin blue is also important; if one is increased at the expense of the other, deficiencies and overlaying become apparent.

Procedures

Method I (critical)

1. Stain in Regaud's iron hematoxylin for the usual times.
2. Rinse in tap water and place the section in picro-orange until the section stains an even yellow and the nuclei are suitably differentiated; (3–5 minutes.)
3. Wash the sections in tap water until the yellow is removed from all elements with the exception of these which are to retain this color. In general only the erythrocytes will be left yellow. (The washing time decreases as the hardness of the water increases and in certain instances when the water is very hard it would be better to use distilled water for this process, in order to have the differentiation under control.)
4. Stain the section in the acid fuchsin mixture. Generally the erythrocytes are the only elements to be left yellow and, as long as they are not stained red, it is best to continue staining in the red dye as long as possible and then to differentiate strongly. (The staining time varies according to the tissue, fixative and the requirements of the individual worker, but will be found to be about 5–10 minutes.)
5. Rinse in 2% acetic acid and transfer to the red differentiator until most of the red has been removed and the various elements are suitably differentiated. (Again the time varies with the needs but will not usually be more than 5 minutes.)
6. Remove the differentiator thoroughly by rinsing in tap water (10 seconds) and then place the section in the anilin blue solution until the connective tissue and other elements are adequately stained. (Average time, 10 minutes; but this time varies with the above-mentioned factors.)
7. Rinse the section in 2% acetic acid and transfer to the blue differentiator for a sufficient time to remove the blue from the red staining elements and for excess to be removed from the connective tissues. (Time varies but should on no account be less than 1–2 minutes.)

8. Remove the differentiator by rinsing in 2% acetic acid; dehydrate rapidly and mount in a neutral balsam, e.g. the recently described synthetic resin balsams. Sections stained by this method and mounted in D.P.X. (Kirkpatrick and Lendrum, 1939, 1941) show no deterioration in twelve months, while sections stained by the original modification four years ago and mounted in this balsam are unchanged.

METHOD II (ROUTINE METHOD APPLICABLE TO LARGE NUMBERS OF SLIDES AT A TIME).

1. Stain in any of the ordinary hemalum solutions for the usual times. Harris' and Mayer's have proved to be useful.
2. Rinse in water and pass directly to picro-acid-fuchsin; (5 minutes frequently sufficient, but this may vary with the fixative used. Sections can be left in this solution indefinitely without coming to any harm, although they will require longer differentiation.)
3. Rinse in 2% acetic acid and place in the red differentiator; (5 minutes' time is the period for which the differentiator has been adjusted in this laboratory.)
4. Rinse in tap water to remove the differentiator and place the sections in anilin blue; (5–10 minutes sufficient.)
5. Rinse in 2% acetic acid and place in the blue differentiator. (The strength of this differentiator has again been adjusted so that adequate results are obtained in 5 minutes.)
6. Remove the differentiator in 2% acetic acid; dehydrate and mount as above.

METHOD III (RAPID ONE SOLUTION METHOD)

1. Stain in hemalum for the normal time.
2. Rinse in water and pass to the "Picro-Mallory" solution for a period depending on the fixative, tissue, etc. Five minutes in this solution gives a satisfactory picture; prolonged staining increases the density without any deterioration in the picture.
3. Rinse in 2% acetic acid and differentiate in the blue differentiator for about a minute. Remove the differentiator in 2% acetic acid.
4. Dehydrate rapidly and mount.

This method is obviously the least critical of the three, but it has the advantages that it is constant in action after fixation in most fixatives, although Zenker's or Helly's fluids are to be preferred. Large numbers of slides can be stained in a dish very rapidly; it

serves a useful purpose in differentiating the various tissues in much the same manner as Mallory's connective tissue stain, and finally from the nature of the stain the individual factor is more or less eliminated by the adoption of standard times.

Notes on the Methods

There is no doubt that the best results are obtained after fixation in solutions containing chromate, and of these fixatives Helly's has proved to be the best for a wide variety of tissues from both man and animals. All the commonly used fixatives, however, are suitable for methods I, II and III if the strengths of the differentiating solutions are adjusted to give the desired results.

All the solutions can be used in staining jars and if the proper precautions are taken remain active over long periods. The main points to be watched are: first, the acidity must be maintained in the anilin dyes, and second, thorough rinsing between the stages as indicated is necessary to keep the staining solutions pure.

The nuclei may seem to be rather faint after staining and differentiating in the picro orange. This is corrected when the sections are stained in the fuchsin which strongly re-inforces the existing nuclear stain.

My thanks are due to Dr. A. C. Lendrum for much helpful criticism and to Mr. W. Penny, Mr. W. Carson and Mr. J. Rennie for their technical assistance and judgment throughout the evolution of these methods.

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THE COMBINED GRAM-PAPPENHEIM STAIN AS MODIFIED FOR FILMS AND PARAFFIN SECTIONS

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A combination of the Gram-Pappenheim stains for the examination of gonorrheal pus, cellular exudate and paraffin sections of formalin-fixed tissues has been described elsewhere (Scudder and Lisa, 1931). The crystal violet solution was made stable for the first time by employing phosphate buffers on the acid side of neutrality, and a stable counterstain was prepared for the first time from National Aniline dyes, ethylated methyl green and pyronin yellowish. Original findings were demonstrated by means of color plate I and II (Scudder, 1931) to show gonococci, pneumococci and cells in smears, and formalin-fixed tissue brought down to water in the usual way. A new color plate is published herewith to show the microscopic appearance of cells, Gram-positive and Gram-negative bacteria, higher bacteria, fungi and spermatozoa in the study of genito-urinary and gynecological cases. The method has a value in the field of medical jurisprudence. Crystals were well demonstrated, especially those resulting from sulfa drug therapy. The National Aniline methyl green batches numbered NG 10, 11, 13 to 19, and their batches of pyronin numbered NP 5 to 10 were found consistently stable. Earlier dyes were found either too purple or too blue for the technic and the most satisfactory dyes were found to require a ripening time of several days and could be prepared in amounts of from 1 to 4 liters and stored indefinitely without preservatives.

HISTORICAL COMMENTS

The first discriminating directions for buffering aqueous crystal violet solution by means of acid phosphates and for the selection of ethylated methyl green and pyronin yellowish dyes to be employed as a counterstain in the Gram technic were made by Scudder (1930–31). Laboratory research was begun in New York City in 1928–30 under auspices of the Biological Stain Commission and the Bellevue-Yorkville Health Demonstration, continued at the City Hospital, Welfare Island, New York City, and published as a monograph (Scudder, 1933) by the Medical Social Service Committee of Greater New York.

An alkaline Gram stain of temporary stability was first employed

in this country by Burke (1921) and the first suggestion of a universal method in which methyl greens and pyronins might be employed in the counterstain for the Gram technic was made by Pappenheim (1903). Sandiford (1937, 1938) published his method for a combined Gram-Pappenheim stain using European and British methyl greens, malachite green and pyronin bluish, calling attention to an earlier paper published by Fauth (1918) in which this combination had been advocated for the examination of smears from cases of chronic gonorrhea. The last three investigators employed alcohols, phenols, and glycerols as aids for dissolving and stabilizing the dye mixtures, but none of them differentiated between ethylated and methylated dyes, the percentage of dye content, the degree of solubility in water, or the "Blau" and "Gelb" designations of Grübler's dyes. Impurities such as free crystal violet or the presence of excess acid went unrecognized, as well as Pappenheim's earlier communication (1901) in which aqueous solutions of methyl green and pyronin had been employed.

The monograph by Scudder (1933) and other papers had established the fact that a new ethylated methyl green dye had been manufactured by the National Aniline Company and that pyronin yellowish was also being supplied by that company for use in the author's technic. These dyes had been found stable. Later research has shown that a dye content of about 76% is characteristic of the best methyl green (ethylated) dyes and that the purest dyes

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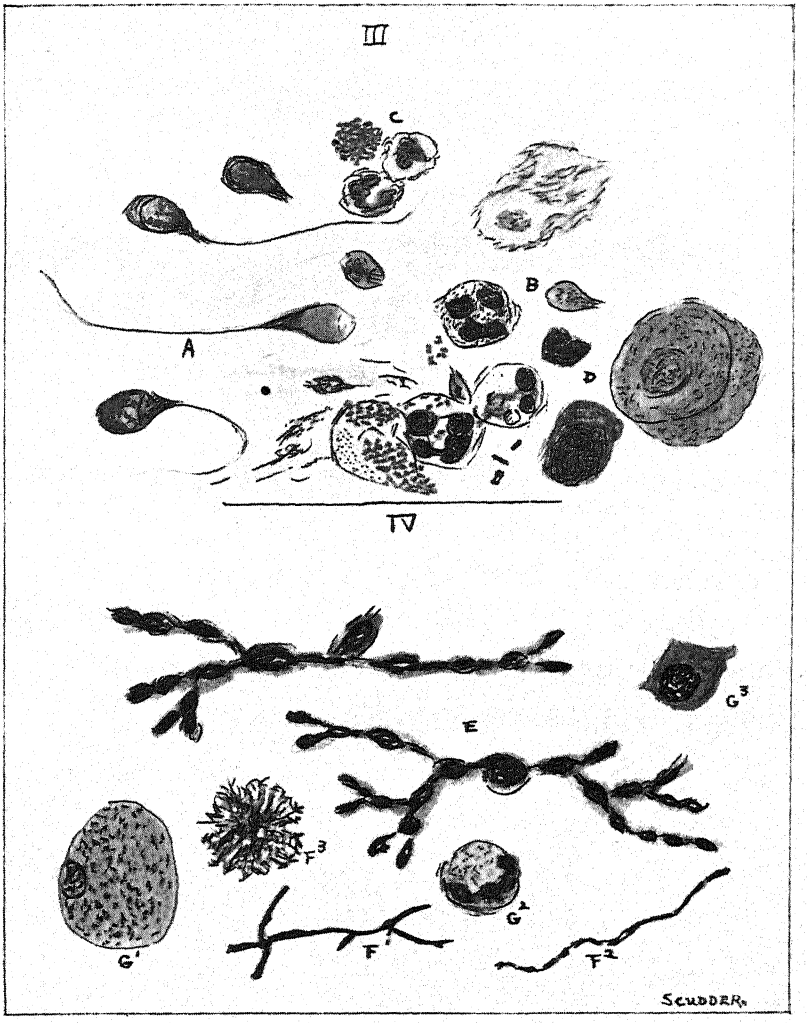
EXPLANATION OF COLOR PLATE[1]

3. Exudate from male cases (Gram differentiation)
   - A. Semen
   - B. Gonorrheal pus with disintegrated spermatozoa
   - C. Colony of gonococci
   - D. Mononuclear cells
4. Exudate from female cases (Gram differentiation)
   - E. Penicillia
   - F. 1 to 2—Actinomycetes culture
     3—Colony of Actinomycetes from axillary node of a guinea pig following inoculation with material from ovarian abscess in human case
   - G. 1-2-3—Different types of pus cells

[1]Plate 1 to 2 was published by Scudder (1931)
Gram differentiation:
1. Stain 3–5 minutes, 1% aqueous crystal violet buffered with acid phosphates, pH 6.6 to 6.9.
2. Flush 1½–2 minutes with alkaline iodine solution (Kopeloff and Beerman formula).
3. Decolorize rapidly, less than 10 seconds in pure technical acetone.
4. Counterstain 1½–2 minutes with National Aniline ethylated methyl green and pyronin yellowish solutions in hot distilled water (Scudder formula).


III
C
A
B
D
IV
E
G3
F3
G1
F1
F2
SCUDDER.

require the longest dissolving and ripening process. Apparently Thomas (1936) had not seen the above publication when he wrote that the dyes employed by Scudder were unstable.

New Gram Stain with Ethylated Methyl Green and Pyronin Yellowish Counterstain

1. *Spread properly selected infectious material in thin films upon the slides, allow to dry, and fix by passing quickly through a flame several times.* Reagents and materials must be of highest purity; slides are chemically clean, dry and free from alcohol or acids. Merchurochrome, sulfapyridine and sulfanilamide are especially inhibitive to accurate staining; some newer drugs such as sulfadiazine do not seem to interfere. Glandular extracts, such as theelin, change the cytology, reduce the number of organisms and render cultures necessary for differential diagnosis.

2. *Flush the crystal violet solution upon the slide 3 to 5 minutes.* This solution is made up, not exceeding 1% in distilled water and stabilized by adding one part phosphate buffer (pH 6.9 or lower) in 10 parts of the crystal violet solution. Merck's Blue Label anhydrous Sorenson's phosphates are employed according to the method of Clark and Lubs. The dye mixture has remained stable for more than 10 years and one gallon amounts have been prepared at a time and stored in brown bottles in dark closets.

3. *Flush the slide with alkaline iodine solution for 1 to 2 minutes (Kopeloff and Beerman, 1922) after decanting the crystal violet.*

4. *Decolorize the slide rapidly in pure technical acetone for 10 seconds.*

5. *Air-dry thoroughly (moisture interferes) and apply the counterstain 1 to 2 minutes.* The slides are washed thoroughly in water and dried preparatory to examination by means of oil immersion lens. The counterstain is prepared in proportion of 0.65 g. of ethylated methyl green and 0.1 g. of pyronin yellowish in 100 ml. of hot distilled water, allowed to age several days or weeks and tested with known controls of bacteria and cells before being employed in routine diagnosis. Where the purity and dye content (76–80% approximate for methyl green and 50% approximate for pyronin yellowish) is known, no modification of formula is necessary.

Summary and Conclusion

Differentiation of wide range is obtained with this new Gram-Pappenheim technic: Polymorphonuclear cells, monocytes, epithelial cells, red blood cells, Gram-positive and Gram-negative bacteria,

fungi, spermatozoa and crystals are clearly defined as shown in Plates 3 and 4 and previously reported by Scudder in 1938. Crystal morphology is especially clear and excess crystals from therapeutic agents may be detected in urine and blood *cultures*. The technic has a place in medicolegal cases. Careful selection of the dyes is necessary and newly prepared solutions should be tested on known controls of Neisserian pus and mixed Gram-positive and Gram-negative bacteria, before being employed in routine diagnosis. Alcohols, phenols, and glycerols are found unnecessary in the counterstain. This counterstain has given as good results when used alone as the best Pappenheim combinations. An alternate counterstain of 0.1% aqueous basic fuchsin may be employed for comparison especially when blood cultures are stained for differentiation of gonococci, meningococci and staphylococci. The reagent solutions may be stored, in 1–4 liter quantities, in cool dark closets.

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NOTES ON TECHNIC

Acetic Methylene Blue Counterstain in Staining Tissues for Acid-Fast Bacilli

When counterstaining tissue sections with methylene blue after a carbol-fuchsin stain for tubercle or lepra bacilli, it is often difficult to achieve a counterstain which is neither too dense nor too light. While alum hematoxylin may be used successfully, this does not stain plasma cells, mast cells, and other bacteria, as does methylene blue.

For a number of years we used a 1:500 methylene blue buffered to pH 3.5 with citric acid and Na_2HPO_4. This was quite successful, but was subject to the mold fermentation of citric acid in aqueous solutions and sometimes went bad.

Recently we tried out a 3-minute stain in 1% methylene blue in 0.5% aqueous acetic acid solution after the usual hot carbol fuchsin, acid alcohol sequence, and secured quite selective nuclear staining with light blue cytoplasm, yellowish orange-red corpuscles, and bright red acid-fast bacilli.

By adding 20% alcohol to the solvent, the growth of organisms may be prevented. However, solutions of methylene blue in 0.5% aqueous acetic acid are relatively stable. They polychrome slowly and slightly. One such two-year-old solution had a spectroscopic absorption maximum of 645 mμ, indicating alteration about as far as azure B.

With this strength (1%) of methylene blue, it is more economical to stain in a staining jar than on the slides. One jar of approximately 150 ml. was used to stain 300 slides over a period of about 10 weeks, with quite satisfactory results throughout.—R. D. Lillie, *Senior Surgeon, U. S. Public Health Service; Division of Pathology, National Institute of Health, Bethesda, Md.*

Orientation of Young Intra-Uterine Embryos for Sectioning

Very young embryos of neural plate and early somite stages of animals such as the dog, cat, rabbit, etc., require careful orientation for sectioning so that the plane of the series will be a favorable one for fruitful study. This orientation may be accomplished by the following procedure:

After the specimen has been hardened by the fixing fluid, carefully cut the muscle coat from the expanded portion of the uterus in which

the embryo lies. Dehydrate the specimen in alcohol and clear in cedar wood oil or some other suitable clearing oil that makes the tissues translucent. While in the oil examine the specimen under a binocular dissecting microscope. Pass a strong beam of light thru the specimen and turn it about with a camel's hair brush. The embryonic area can be distinguished as a denser line stretched across the uterine cavity in the manner of a plane cutting a segment from a sphere near its surface. Slice off the side of the uterine wall opposite the embryonic area with a razor blade and the exact axis of the embryo can be observed under the microscope and marked as the specimen is embedded in paraffin. This method eliminates the danger of mechanical injury and damage by diffusion currents.—MELVIN C. GODWIN, *Department of Histology and Anatomy, West Virginia University, Morgantown, W. Va.*

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

COWDRY, E. V. **Microscopic Technique in Biology and Medicine.** 6 x 9¼ in. 206 pp. Cloth. Williams and Wilkins Co., Baltimore, Md. $4.00.

The author of this book has adopted the encyclopedic method of presenting the subject. So far as the reviewer is aware, this is the first time this method of discussing microtechnic has been adopted since the large encyclopedias of Ehrlich and of Krause. Really, however, this new book is hardly to be compared with the earlier German works. The author has endeavored—far from presenting, in German style, all available knowledge on the subject—to give in concise form the information of most practical value to the worker in microtechnic. Because of the brief and concise method of presentation, the book is small enough to find its place either on the desk or the laboratory table—can, in fact, almost be carried in the pocket. It ought to prove distinctly useful.—*H. J. Conn.*

PHOTOMICROGRAPHY

BAKER, JOHN R. **Tube-length in photomicrography.** *J. Roy. Micr. Soc.*, **62**, 112–5. 1942.

When a microscope is focused visually and then a camera is applied without further adjustment of focus, the object (or part of the object) seen by the eye is not in focus on the ground glass of the camera. The method here recommended for bringing the image into focus on the ground glass applies to the photomicrography of objects mounted in balsam or other medium of similar refractive index, and covered by cover glasses of the thickness for which the objective used is corrected. It is recommended that, after visual focusing, further focusing for the camera be achieved simply by extending the draw-tube of the microscope. The amount of extension necessary depends wholly on the eyepiece, and once this amount is accurately determined for a particular eyepiece, the ground glass may be discarded. In using this method, it is essential that the camera extension be fixed, the microscope held in sockets, and the photomicrographic lamp clamped in position.—*C. E. Allen.*

MICROTECHNIC IN GENERAL

FLEMING, W. D. **Synthetic mounting medium of high refractive index.** *J. Roy. Micr. Soc.*, **63**, 34–7. 1943.

Mounting media whose refractive index is close to that of glass serve well for stained specimens. But for colorless specimens such as diatoms, visibility is enhanced if the refractive index of the medium is higher than that of the object. Directions are given for the preparation of a synthetic resin designated *Naphrax*, whose refractive index is 1.7–1.8. That of diatoms is approximately 1.4.

In a glass vessel of 1500–2000 ml. capacity, preferably provided with a motor-driven stirrer and arranged for chilling in cracked ice, 200 ml. formalin is poured over 200 g. naphthalene. To the chilled mixture is added, very slowly and with constant agitation, a well-chilled mixture of 600 ml. glacial acetic acid and 300 ml. concentrated H_2SO_4 (sp. gr. 1.84). The temperature is held below 10° C. for the first 6 hr. Stirring is important during this period. Then the temperature is allowed to rise naturally to room temperature during 6–12 hr., stirring being maintained at least intermittently. The container is next surrounded by a water bath and the temperature of the mixture is raised gradually to 60° C., at which point it is maintained with agitation until a sample of the yellow resin, removed and washed in water, is brittle at room temperature.

Purification (which is important) is accomplished by washing the mass of crude resin in several changes of hot water, kneading the mass in the water with a heavy glass rod, then washing in two changes of warm 5% Na_2CO_3.

Next it is chilled and ground to a fine powder in a chilled mortar, pieces of ice being mixed with the resin to keep it brittle. It is washed by decantation in cold 5% Na_2CO_3, then in ice water. The powder is dried on a filter and washed with several changes of cold 95% alcohol. It is further washed by kneading or stirring the mass with a heavy glass rod in several changes of boiling 95% alcohol, then in 1 or 2 changes of boiling absolute alcohol. The mass is chilled, powdered in a dry, chilled mortar, washed with absolute alcohol, dried in air, and dissolved in pure xylene, toluene, or benzene. Dilution with the solvent continues until no more precipitate develops on standing at a low temperature. To the thin solution a small amount of anhydrous Na_2CO_3 is added and the solution is allowed to stand at least 24 hr. in an ice box, with frequent shaking during the early part of the period. It is decanted and filtered through the finest filter paper available. Finally the solvent is evaporated at a temperature not above 100° C. until the resin is a moderately firm mass.

It is advisable to add 1% of a plasticizer (dibutyl phthalate is recommended) to the pure resin.—*C. E. Allen.*

JELLEY, EDWIN E. **A review of crystallographic microscopy.** *J. Roy. Micr. Soc.*, **62**, 93–102. 1942.

The author gives a brief history of the development of the polarizing microscope and its accessory apparatus as used in optical crystallography, with reference to petrography and chemical microscopy.—*C. E. Allen.*

WATSON, J. M. **The ripening of Ehrlich's hematoxylin.** *J. Roy. Micr. Soc.*, **63**, 20–5. 1943.

Three methods, each involving the addition of an oxidizing agent, have been devised which make it possible to use Ehrlich's hematoxylin immediately after its preparation. All gave results, in selectivity and contrast as nuclear stains, equal if not superior to the naturally ripened stain, and of much greater reliability and consistency. Formula 1 stained more rapidly than either of the others.

FORMULA 1: 0.1 g. $KMnO_4$ dissolved in 100 cc. of 2% aqueous ammonia alum; 100 ml. 2% solution of hematoxylin in absolute alcohol; 10 ml. glacial acetic acid; 100 ml. glycerin.

FORMULA 2: 100 ml. 2% aqueous ammonia alum; 100 ml. 2% hematoxylin in absolute alcohol; 100 ml. glycerin; 10 ml. glacial acetic acid; 1.3 g. chloramine T.

FORMULA 3: 100 ml. 2% aqueous ammonia alum; 100 ml. 2% hematoxylin in absolute alcohol; 100 ml. glycerin; 40 ml. glacial acetic acid; 2 g. barium peroxide.—*C. E. Allen.*

WICKS, L. F., and SUNTZEFF, V. **Glyoxal, a non-irritating aldehyde suggested as a substitute for formalin in histological fixations.** *Science*, **98**, 204. 1943.

Glyoxal, or oxalic aldehyde, has been found to be a satisfactory substitute for formalin. A 2% glyoxal solution is equivalent to 10% formalin (4% formaldehyde) in fixing small pieces of tissue. Up to 10% glyoxal may be used for large masses of tissue, such as an entire human brain.

Glyoxal is available as a 30% to 40% aqueous solution, impure and too acidic for immediate use. It should be diluted to 10% and $CaCO_3$ added until effervescence stops, then filtered by suction through a rapid crepe paper. The solution will be faintly acid to litmus and should remain so.

The fixing solution has only a weak odor and is non-irritating. Results on tissues stained with hematoxylin-eosin are indistinguishable from those obtained with formalin fixed tissues.—*T. M. McMillion.*

DYES AND THEIR BIOLOGICAL USES

COPLEY, A. L., and WHITNEY, D. V. **The standardization and assay of heparin by the toluidine blue and azure A reactions.** *J. Lab. & Clin. Med.*, **28**, 762–70. 1943.

In view of the increasing use of heparin, a direct chemical assay method is desirable. The two following methods are described.

Method I: Prepare a 1:1000 solution of toluidine blue. (The dye used was that produced by the National Aniline Chemical Co. and certified under the number NU-3 and its composition required 1.6949 g. per 1000 ml. to yield a

1:1000 dilution of pure dye.) Prepare also a 1:1000 solution of azure A (certified as NAz7) by dissolving 1.1494 g. in 1000 ml. of water. In a series of tubes place 1, 2, 3, 4 and 5 Murray-Best units of heparin (1 unit equals 0.01 g. of the crystalline Ba salt of heparin), and make each up to 5 ml. with water. Let stand 15 min. and centrifuge at 3600 r.p.m. for 15 min. Decant the supernatant liquid and compare in a Duboscq colorimeter with 6 ml. of a 1:6000 solution of toluidine blue or azure A. Compute the residual dye in the supernatant liquid and subtract from 1000 γ (the original dye concentration) to get the amount of dye reacting with the heparin present. Experimentation gave an average of 140 γ of either dye reacting with 1 unit of heparin. Since the heparin unit was considered rather low, it was decided to establish the unit of heparin as that amount which reacts with 150 γ of dye.

Method II, carried out as follows, seems more accurate than method I: Prepare a 1:2000 solution of either dye. To this solution add 0.05 ml. increments of heparin solution, with swirling, from a burette, until precipitation starts, then drop by drop until a distinct blue equal to about 1:40,000 of the dye is observed among the heparin floccules. It was demonstrated that 150 γ of dye precipitates 1 unit of heparin. The test is reproducible with an accuracy of 5%, and is suggested as a practicable method for assaying heparin.—*John. T. Myers.*

ROTHMAN, STEPHEN, and FLESCH, PETER. **Isolation of an iron pigment from human red hair.** *Proc. Soc. Exp. Biol. & Med.*, 53, 134–5. 1943.

An amorphous brownish powder with a metallic sheen, insoluble in water and organic solvents but soluble in alkalis, was extracted from bright red human hair. The purplish pigment, the reversible color change with a change in pH, and the absorption spectrum suggest that this substance belongs in the group of complex phenolic iron compounds. The phenolic OH group may be attached to a heterocylic ring containing nitrogen.—*M. S. Marshall.*

MICROÖRGANISMS

BEAMER, P. R., and STOWELL, R. E. **The use of tertiary butyl alcohol in bacteriologic staining procedures.** *J. Lab. & Clin. Med.*, 28, 1599–602. 1943.

Tertiary butyl alcohol may be substituted for ethyl alcohol in the Gram and Ziehl-Neelsen staining methods. The following technic is satisfactory: Apply crystal violet solution (2 g. crystal violet in 20 ml. of tertiary butyl alcohol) for 10 sec., wash in tap water, Gram's iodine solution 10 sec., wash in tap water, 80% tertiary butyl alcohol 60–90 sec., wash in tap water, counterstain with safranin. For the Ziehl-Neelsen technic for acid-fast bacilli, use 5% HNO_3 in 50–80% tertiary butyl alcohol for decolorization. Tertiary butyl alcohol is readily available and reasonably priced. It may be employed if the present reagents become unobtainable or prohibitive in price.—*John. T. Myers.*

BRAY, J., and KING, E. J. **The phosphatase reaction as an aid to the identification of micro-organisms using phenolphthalein phosphate as substrate.** *J. Path. & Bact.*, 55, 315–20. 1943.

The incorporation of phenolphthalein phosphate (prepared by King, E. J., *J. Path. & Bact.*, 55, 311–4, 1943) in a solid medium such as Dorset's egg, which minimizes the diffusion of color, was found to provide a striking means of demonstrating the action of bacterial phosphatases, especially when the plate cultures were exposed to ammonia vapor. *Streptococcus pyogenes* reacted much more strongly than did pneumococci; "Certain of the diphtheroids were easily distinguished from *C. diphtheriae* by the intensity of their reaction"; and other differences were observed among clostridia, intestinal bacteria, and species of *Hemophilus*. A striking color plate illustrates some of these differences.—*S. H. Hutner.*

CROSSMAN, GERMAIN, and LOEWENSTEIN, ERICH. **Demonstration of tubercle bacilli in tissue by fluorescence microscopy.** *J. Lab. & Clin. Med.*, 28, 1349–54. 1943.

The following method is recommended: Fix tissue in 10% neutral formalin for 12–24 hr., dehydrate, embed in paraffin, and cut sections 6–10 μ thick. Remove the paraffin completely (it is fluorescent), pass through alcohol series to water and place in auramine (auramine O, National Aniline, 0.1 g., distilled

water 100 ml., U.S.P. liquid phenol 4 cc.), steam gently for 3–5 min. (room temperature 30 min., 37° incubator 15 min., or 55° incubator 10 min.), wash in several changes of distilled water and decolorize with alcohol containing 0.5–1.0% HCl till quite colorless (about 45 sec.). Wash in distilled water. Stain 30 sec. in 0.1% aqueous methylene blue (National Aniline, Certification No. 18). Rinse in distilled water, dehydrate, clear in xylene and mount in xylene-clarite. Use a microscope equipped with a mercury vapor lamp with an iris diaphraghm, an ultraviolet transmitting filter and a dark-field stop. Place non-fluorescent sandalwood oil or Shillaber's immersion oil between the slide and the condenser. Enclose 3 sides of the microscope with a shield to exclude extraneous light. An aluminumized mirror is preferable to a silvered one. Tubercle bacilli appear as yellow, thin, luminous, slightly curved rods on a dark background.—*John T. Myers.*

KNAYSI, G., and MUDD, S. **The internal structure of certain bacteria as revealed by the electron microscope—a contribution to the study of the bacterial nucleus.** *J. Bact.*, 45, 349–59. 1943.

Treatment of bacteria with 0.02% aqueous $NaHCO_3$ solution for about 2 hr. before films were prepared for study in the electron microscope reduced the opacity of the cells without affecting that of the internal granules; 5 hr. in 2% by vol. HNO_3 in water was also effective but started to dissolve the granules; 5 hr. in 10% HCl in water left the bacteria completely opaque. Granules believed to be nuclear were demonstrated in many but not all the species studied. In some bacteria granules were hard to demonstrate in young cultures even tho they were quite distinct in older cultures.—*V. Kavanagh.*

KRAJIAN, A. **A new and rapid staining method for Gram positive and Gram negative organisms in frozen and paraffin sections.** *J. Lab. & Clin. Med.*, 28, 1602–6. 1943.

The following technic is recommended: Prepare 7–10 μ, mounted frozen sections. Stain 3 min. with alkaline methylene blue (Loeffler). Wash in tap water and dehydrate rapidly with three applications of ethyl or isopropyl alcohol. Differentiate rapidly with creosote-xylene (1 part creosote and 2 parts xylene), agitating for 5–10 sec. Pour off and apply creosote-fuchsin (2.5 ml. of 6% alcoholic basic fuchsin added to 50 ml. of creosote-xylene), agitating 15–20 times, and changing the solution once. Blot and apply creosote-xylene until the excess of red color is gone. Blot, clear 2 min. in xylene, and mount in gum dammar. For paraffin sections deparaffinize with 2 applications of xylene and 2 applications of absolute ethyl or isopropyl alcohol, bring down to tap water and proceed as above. The method is simple, rapid and demonstrates all bacteria including altered forms of Gram positive organisms.—*John T. Myers.*

LEVIN, MAX. **The effect of concentration of dyes on differentiation of enteric bacteria on eosin-methylene blue agar.** *J. Bact.*, 45, 471–5. 1943.

The original eosin-methylene-blue agars were made with Grübler dyes which had less dye content than have the American dyes now in use. This has resulted in a disproportionate increase in the amount of methylene blue so that the media have a blue tinge instead of the amber desired; the colonies of the Salmonella and typhoid-dysentery groups take an atypical blue stain. To avoid these difficulties, the ratio of eosin to methylene blue should be approximately 6 to 1 and the pH of the medium should not exceed 7.4. These conditions are met by preparing the agar as follows: to 100 ml. of the melted basal medium (15 g. agar, 10 g. peptone, 2 g. K_2HPO_4 per liter) add 5 ml. of a 20% sterile solution of lactose, 2 ml. of a 2% aqueous solution of eosin-yellowish, and 2 ml. of a 0.325% aqueous solution of methylene blue (American dyes, sources not given).—*V. Kavanagh.*

McCLUNG, L. S. **On the staining of yeast spores.** *Science*, 98, 159–60. 1943.

The author describes an improved method for staining yeast spores with malachite green. Solutions needed are: 1% malachite green dissolved without heating in 1% aqueous phenol; 0.5% aqueous safranin. The procedure is as follows: Prepare smear on a clean slide, air dry and fix lightly with a Bunsen flame. Cover entire slide with malachite green solution and steam 2 min. (do not boil) with a low Bunsen flame. Wash 1 min. in a gentle stream of tap-water; counterstain 30 sec. with aqueous safranin. Yeast spores should appear green, asci a deep pink.—*T. M. McMillion.*

STAIN TECHNOLOGY

VOLUME 19 APRIL, 1944 NUMBER 2

DEMONSTRATING THE OSSEOUS SKELETON OF HUMAN EMBRYOS AND FETUSES

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ABSTRACT.—Transparent human embryos and fetuses whose osseous skeletons are stained in toto by alizarin red S are successfully prepared when the KOH clearing of the soft tissues and the alizarin staining of the bones are performed simultaneously instead of independently. This modification minimizes the possibility of macerating and staining the soft tissues. Fetuses over 50 mm. CR length are skinned, eviscerated, decerebrated, defatted by dissection, fixed in 95% alcohol, bleached in H_2O_2, cleared and stained simultaneously in an aqueous solution of KOH (from 2% to 10% depending upon the size of the specimen) and .0001 to .00005% alizarin red S (solution has a pale lavender color). This solution is changed periodically to maintain the concentration of the KOH until the clearing of the tissues is complete and of the alizarin until the bones are properly stained. Tissues are dehydrated in increasing concentrations of glycerin and stored in white glycerin plus thymol.

INTRODUCTION AND HISTORY

This is a report of a tissue clearing and a bone staining technic, demonstrating the *in situ* osseous skeleton, in which the KOH clearing and the alizarin red S (sodium 1,2-dihydroxyanthraquinone-3-sulfonate) staining steps of the modification are executed simultaneously instead of independently. The clearing of the soft tissues and the staining of the bones can be effectively controlled and the maceration and the staining of the soft tissues can be easily prevented in this modification. Human embryos and fetuses from 25 mm. crown-rump (CR) length through six fetal months have been successfully cleared and their bones stained red by this method. The fetuses over six lunar months did not clear well in all regions. The problems of fixing, differentially staining, dehydrating, clearing and storing specimens, here omitted, are adequately discussed in the literature.

Lemnius (1581), Belchier (1736) and others (see Cameron, 1930; 1932 and Ercoli and Lewis, 1943) recognized the property of madder to stain bone red. Beale (1858), who prepared the first transparent human embryo in 1853 with NaOH and glycerin, Schultze (1897), who described the KOH method of clearing embryos, and Mall (1906), who introduced Schultze's technic in America, are the pioneers in the strong alkali and glycerin clearing technics. Lundvall, (1905), Dawson (1926), Hollister (1934) and many others have perfected modifications of the technic using KOH for clearing the soft tissues and alizarin red S for staining the bones.

The fixation of the alizarin dye is ascribed to its chemical affinity for recently deposited calcium salts (Macklin, 1917, Cameron, 1930 etc.). However Ercoli and Lewis, (1943) claim that alizarin red S fixation occurs by a process of adsorption (physical phenomenon) between existing calcium phosphate and dye anions, rather than by the supposed formation of calcium alizarate or a reaction with free calcium ions (chemical phenomenon).

Procedure

1. All human fetuses larger than 50 mm. CR are skinned, eviscerated, decerebrated through a slit in the frontal fontanelle and defatted by dissection. Acetone does not effectively remove the fat in large fetuses. Some of the neck, facial, pelvic and other heavy musculature should be removed from the large fetuses. Experience is the only guide as to the amount of dissection required. Embryos and fetuses under 50 mm. CR may be eviscerated. They are fixed in 95% alcohol for two weeks to two months depending on the size of the specimen. As the alizarin red S staining affinity is dependent on the calcium salts in the bone (Cameron, 1930), the decalcifying properties of neutral or slightly acidic formalin may possibly render this preservative unsatisfactory for the alizarin staining of slightly calcified bony structures.

2. Material is bleached in a mixture of 9 volumes of 95% alcohol to one volume of 3% aqueous H_2O_2, and returned to 95% alcohol until ready for clearing.

3. Fetuses are cleared and stained *simultaneously* in a solution of alizarin red S[1] in aqueous KOH (preferably in distilled water; see Hollister, 1934). The concentration of the dye should be 0.0001 to 0.00005% (which has a pale lavender color); and the strength of the KOH should be varied as follows:

[1]Alizarin red S, used in this technic, was alizarine technical which was obtained from George T. Walker Co. Minneapolis, Minnesota.

a. For fetuses up to 150 mm. CR length, 2 to 4% KOH.
b. For fetuses over 150 mm. CR length, 4 to 10% KOH.

The solutions are changed periodically in order to maintain the concentration of KOH and the alizarin red S in the solutions until the tissues are adequately cleared and the bones are well stained. Adequate clearing (bones clearly visible) may take from 3 to 4 days in small specimens to 2 to 3 weeks for six-month fetuses. When the bones are adequately stained, if clearing is not complete, it may be continued in an aqueous solution of KOH or in a dilute glycerin solution (10% glycerin in distilled water) of KOH.

In previously published technics the specimens are first cleared and then the bones are stained in a solution of alizarin red S in KOH. While staining the skeleton in this solution, the extraosseous tissues continue to clear. If the clearing of a specimen has been carried too far during the clearing step of the technic, maceration of the soft tissues often results while the bones are staining. In this modification the process of clearing is completed at the same time as the staining of the bones, or slightly later, and thus the clearing step can be stopped at the proper time and yet have the bones stained intensely red. When low concentrations of alizarin red S are used for staining the bones, the bones stain intensely red and the extraosseous tissues do not stain at all. Thus the clearing of the soft tissues and the staining of the bones can be effectively controlled, and the maceration and the staining of the soft tissues can be easily prevented.

4. Specimens are finally cleared and dehydrated in increasing concentrations of glycerin. Specimens, cleared in glycerin, are easily dissected, do not undergo shrinkage, keep indefinitely and clear completely.

5. For storage, white glycerin, in which a crystal of thymol is dissolved to prevent mold from growing, is used. Specimens should be stored in darkness although some exposure to light does not visibly destain the bones.

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VARIOUS OIL SOLUBLE DYES AS FAT STAINS IN THE SUPERSATURATED ISOPROPANOL TECHNIC

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SUMMARY.—Oil red O (xylene-azo-xylene-azo-β-naphthol), oil red 4B or EGN (xylene-azo-toluene-azo-β-naphthol) and Sudan red 4B give somewhat deeper orange red or red fat stains and more stable dilute isopropanol solutions than Sudan IV. Sudan II gives brighter orange-yellow fat stains and stronger stable dilute isopropanol solutions than Sudan III. Satisfactory brownish red dyes as to intensity and stability of their dilute isopropanol solutions are Sudan brown, Sudan brown 5B, and oil brown D.

Following previously reported work[1] on the relative efficiency of a number of oil soluble dyes as fat stains when used in dilute acetone methanol solutions, Lillie and Ashburn[2] reported on the use of supersaturated solutions in 50 to 60% isopropanol as fat stains. In that report favorable comments were made on oil red 4B and Sudan brown as substitutes for Sudan IV.

Previously I had accepted the identification of oil red O as *m*-xylene-azo-β-naphthol, given under No. 73 in the Colour Index[3] and accepted by Conn[4], but preferred the name Sudan II for this dye. The dye so named in the first report[1] was obtained from the Commonwealth Color and Chemical Company and from the Hartman Leddon Company, both of whom have identified the samples they furnished as C.I. No. 73 or Sudan II, though the first company named their sample brilliant oil scarlet B concentrated, while the second called both of theirs oil red O. National Aniline Division has recently furnished another sample of Sudan II (C.I. No. 73) under the name oil scarlet 6G, with the definite assurance that it is C.I. No. 73.

The National Aniline Division now gives the information that the dye supplied by *them* as oil red O is not, and never has been C.I. No.

[1]LILLIE, R. D. (In press.) Study of certain oil soluble dyes for use as fat stains. J. Tech. Methods; accepted September 1, 1942.

[2]LILLIE, R. D., and ASHBURN, L. L. 1943. Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degenerations not shown by Herxheimer technic. Arch. Path., **36**, 432–5.

[3]ROWE, R. M. 1924. Colour Index. Soc. Dyers and Colourists, Bradford, Yorkshire.

[4]CONN, H. J. 1940. Biological Stains, *4th Ed.* Biotech Publications, Geneva, N. Y.

73, and that the statement in the Colour Index that their oil red O was a synonym of Sudan II is erroneous. Actually their oil red O is a disazo dye similar to Sudan IV, namely *p*-xylene-azo-*p*-xylene-azo-β-naphthol and even more similar to the oil red 4B mentioned above, which is *p*-xylene-azo-*o*-toluene-azo-β-naphthol. This latter dye is also sold by the National Aniline Division as oil red EGN.

Since French[5] specified in his original report that the oil red O used by him was obtained from National Aniline and that it had a greater depth of color than Sudan III (which Sudan II has not), it is evident that the dye referred to was *p*-xylene-azo-*p*-xylene-azo-β-naphthol and not C.I. No. 73.

The purpose of the present report is to compare a number of oil soluble dyes with Sudan IV, using the new supersaturated isopropanol technic[6]; 35 samples of oil soluble dyes were used, tabulated in Table 1. Saturated stock solutions of all were made in 99% isopropanol, using 200 to 500 mg. per 100 ml. For use all were diluted to 60% by addition of 4 ml. water to 6 ml. stock solution. These dilutions were allowed to stand 5 to 10 minutes, then filtered, and, if further turbidity or precipitation appeared in the first half hour, they were again filtered. Frozen sections of adrenal cortex were stained 10 minutes in these dilutions during the period between 30 minutes and 4 hours after dilution, and another series 24 hours or more after dilution. When fat staining was poor, other sections were given an hour's exposure to the stain. After the fat stain all sections were transferred to water and counterstained 5 minutes in acid hemalum,[7] which had been diluted with 4 vol. of 2% acetic acid. (This is the same concentration as Mayer's original formula, undiluted.) They were then blued in 1% aqueous Na_2HPO_4, rinsed and mounted in modified Apathy syrup. Results are presented in Table 1. There are also given certain spectroscopic data serving to help identify the samples. The value d $\times$ D (i.e. dilution times optical density at the absorption maximum) is inserted as a crude measure of relative solubility in 99% isopropanol. It must be regarded as a crude measure, owing to the variations in composition of the various dyes.

In general, pleasing deep orange red to red colors are given by the Sudan IV, oil red 4B and EGN, oil red O, Sudan red BB and Sudan red 4B. Some of the Sudan IV samples show rather rapid precipitation of the dye and consequent impairment of staining; others appear to give fairly stable solutions. The others of this group have some-

[5]French, R. W. 1926. Fat stains. Stain Techn., **1**, 79.

[6]See footnote 2.

[7]Lillie, R. D. 1942. An improved acid hemalum formula. Stain Techn., **17**, 89–90.

TABLE I. SPECTROSCOPIC AND STAINING DATA ON VARIOUS OIL SOLUBLE DYES.

C.I. NO.	NAME, MANUFACTURER AND LOT NUMBER	λ	W	m	R	d·D	COLOR OF FAT	GRADE OF 10′ STAIN FRESH	GRADE OF 10′ STAIN DAY OLD	REMARKS
73	SUDAN II, CO#26871	498	41	498	1.42	187	ORANGE YELLOW	+++	+	+++ 20′
73	" HL (A)	494	40	498	1.40	226	" "	+++	+	+++ 20′
73	" HL (B)	496	41	498	1.40	168	" "	+++	+	+++ 20′
73	" NA#10547	494	41	496	1.55	456	" "	++++	+++	
81	SUDAN BROWN, NIH#6.942	492	94	477	1.20	39	DEEP RED BROWN	+++	+++	
82	NAPHTHYLAMIN BORDEAUX, NIH#5.2242	491	60	481	1.39	22	ORANGE BROWN	++	+	
113	SUDAN R, NA#9317	500	40	504	1.22	109	ORANGE YELLOW	++	++	
248	SUDAN III, CB#CY-5	504	39	508	1.12	13	BRIGHT ORANGE	+	+	
248	" CB#CY-6	508	37	508	1.14	26	" "	++	++	
248	" GR	506	42	508	1.12	48	" "	+++	+++	
248	" KY-1	512	44	510	1.07	24	" "	++	+	
248	" NA#NY-6	512	40	510	1.08	16	" "	+++	+++	
248	" NA#NY-7	502	35	502	1.30	17	" "	+++	+++	
248	" NIH#11.2643	505	36	504	1.24	5	ORANGE	±	±	+ 1hour
258	SUDAN IV, CB#CZ-5	512	39	512	1.04	50	ORANGE RED	+++	±	+ 20′
258	" CB#CZ-7	521	41	519	0.96	26	" "	+++	−	∓ 20′
258	" HL#LZ-3	512	37	513	1.04	61	" "	+++	++	
258	" HL#LZ-4	514	35	514	1.03	53	" "	+++	+++	
258	" NA#NZ-11	514	39	517	1.01	37	" "	+++	±	+20′
258	" NA#NZ-13	516	39	518	0.97	46	" "	+++	++	
	OIL RED 4B, CO#26870	513	43	515	1.03	64	DEEP ORANGE RED	+++	++	
	OIL RED EGN, NA#123063	522	37	521	0.93	31	DEEP RED	+++	+	
	" NA#165113	518	29	515	0.99	68	" "	++++	++	
	OIL RED O, NA#BC40105	516	38	517	0.97	128	" "	+++	++	+++ 20′
	" NA#10517	521	37	515	0.99	63	" "	++++	+++	+++ 20′
	" NA#BC40104	521	36	518	0.97	57	DEEP ORANGE RED	++++	++++	
	OIL BROWN D, NA#104960	522	63	514	0.99	46	DEEP RED BROWN	++++	++++	
	OIL BROWN M, NA#9259	490	53	487	1.60	35	BROWN	++	++	
	OIL BROWN Y, NA#7356	454	48	446	2.23	142	BROWN ORANGE	++	++	
F.P. NO.	AZO OIL BLACK B, NA#116739	568	57	568		28	DEEP BLUISH RED	±	±	+ 1hour
177	SUDAN BLUE G, GD#S.0.49	640	21	639		8	BLUE GREEN	∓	−	
178	SUDAN BROWN 5B, GD#S.0.49	478	56	482	1.31	53	BROWN RED	++++	++++	
182	SUDAN RED BB, GD#S.0.49	515	37	517	0.99	14	RED	++	+++	
	SUDAN RED 4B, GD#S.0.49	517	39	518	0.96	50	"	++++	++++	
	SUDAN CORINTH 8, GD#S.0.49	520	63	519	0.98	51	BROWN RED	+++	++++	

C.I. NO. = COLOUR INDEX NO., F.P. NO. = FOREIGN PROTOTYPE NO., λ = ABSORPTION MAXIMUM IN mμ., W = WIDTH IN mμ OF ABSORPTION BAND OF OVER 90% MAXIMUM DENSITY (D)., m = MEDIAN OF FOREGOING ABSORPTION BAND, R = RATIO: D500/D530 = CONN'S E500/E530, d·D = DILUTION (d) X DENSITY (D) AT λ OF SATURATED ISOPROPANOL SOLUTION DILUTED IN 95% ETHANOL, ′ = MINUTES. CO = COMMONWEALTH COLOR AND CHEMICAL CO., HL = HARTMAN LEDDON CO., NA = NATIONAL ANILINE DIVISION, NIH = NATIONAL INSTITUTE OF HEALTH, CB = COLEMAN AND BELL CO., GR = GRUEBLER, GD = GENERAL DYESTUFFS CORP.

what deeper orange red or purer red colors, and the 60% isopropanol solutions still stain well 24 hours after dilution. The solubility of Sudan III in isopropanol appears to be too low to give consistently good stains, but the solutions appear to be fairly stable after dilution. However, if a lighter orange yellow stain is desired, for instance to contrast with the blue-black myelin of the Weigert iron hematoxylin method, Sudan II gives good solubility, quite stable dilute isopropanol solutions, and a sharp bright orange yellow color. The color tone of the browns is not pleasing to some observers, but Sudan brown, Sudan brown 5 B (foreign prototype No. 178 in the Yearbook of the American Association of Textile Chemists and Colorists) and oil brown D (constitution not published) give satisfactory color intensities and sharp staining. Sudan R, naphthylamine bordeaux, oil brown M, oil brown Y, and Sudan corinth B give orange or brown tones of lesser intensity and brilliance, though their dilute isopropanol solutions still stain well after 24 hours. Azo oil black B gives good color but requires prolonged staining, while Sudan blue G and Victoria blue B base were unsatisfactory.

Since Sudan III showed variation in color from orange to orange red in various samples, and concurrent variation in position of the spectroscopic absorption band from a median of 501.9 to 510.4 mμ (Sudan IV ranged from 512.0 to 518.7), it was determined to synthesize a sample from the purest available aminoazobenzene and β-naphthol. The resultant sample was apparently less soluble in isopropanol than any of the commercial samples and gave an absorption band median at 504.0 mμ. It is probable that the redder commercial samples are more or less contaminated with the homologous aminoazotoluene derivative.

Acknowledgment is due to Medical Technician Roland Faulkner for technical assistance.

MICROSCOPY WITH PLASTIC SUBSTITUTES FOR COVER GLASSES

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ABSTRACT.—To determine whether plastic substitutes for cover glasses on microscope slides affect the performance of the microscope, their optical constants were determined. The plastic covers and glass cover glasses were mounted also on silvered slides to form Star Test Plates which were studied by competent observers. The thicker plastic cover glasses, now on the market, are satisfactory when mounted to give a plane surface. Optically inhomogeneous materials, of irregular thickness, those that curl or do not have plane surfaces, adversely affect the performance of the microscope and should not be used. Since the substitutes are softer than glass they must be protected from abrasion. It is recommended that thicknesses of 0.18 mm., and none outside of a range of 0.12 to 0.20 mm. be used. For critical observation with unimmersed objectives of high aperture, best results are obtained with the correction collar set at the position corresponding to the actual thickness of the cover slip, just as would be done with glass cover glasses.

Plastic sheeting has been recommended as an inexpensive substitute for glass cover slips in microscopic study. The suitability of these substitutes from a chemical and mechanical viewpoint has been described. This paper reports on the affect of these materials on the performance of the microscope.

Little information on the optical properties of the plastic covers is available.[1] Microscopists have reported their use with varying satisfaction. Kenohan (1928) used moisture proof Cellophane (DuPont) 0.25 mm. thick with Wright's medium. Suntzeff and Smith (1940, 1941) recommended VuePack (Monsanto) 0.17 mm. thick for mounts with balsam and isobutyl methacrylate, but withdrew their recommendation on finding that their preparations faded. Cellophane 0.022 mm. thick was preferred to an isobutyl methacrylate film by Bucholtz (1940).

Prevost (1942) used Cellophane mounts sealed with petrolatum for temporary preparations for dark field examination. Cellophane cover slips (No. 88 gauge) cut from rolls $\frac{7}{8}''$ wide were found satisfactory by Pons (1942) after nine months. Tissues imbedded in

[1]See Pfund (1939) and Strain *et al.* (1939).

No. 120 Cellophane, or Nos. 005, 0125 and 020 VuePak, were handled conveniently as a ribbon by Minckler (1942). Russell (1942) reported VuePack inexpensive but unsuited for permanent mounts. Elias (1942) found plastic sheets helpful when handling large numbers of sections.

A thin film of plastic evaporated onto a preparation has been used instead of a cover slip. Such films are too thin to be satisfactory optically, or to be tested in this study, although they do provide mechanical protection. Microscope objectives corrected for use without a cover glass should be used for best results on preparations covered with such thin films.

TABLE 1.—OPTICAL CHARACTERISTICS OF GLASS AND PLASTIC COVER GLASSES

Material	N_D	Thickness t mm.	t/N_D	Correction Collar Setting	
				(I)	(II)
Cellulose Acetate A..	1.488	0.14	0.094	13	13
Cellulose Acetate B..	1.491	0.18	0.121	18	18
VuePak.....	1.498	0.13	0.087	13	12
Cellophane.....	1.535	0.03†	0.020	*u*	*u*
Vinylite A.....	1.528	0.16	0.105	16	17
Vinylite B.....	1.528	0.14	0.092	(15)*	(13)*
Pliofilm.....	1.54	0.04†	0.026	*u*	*u*
Glass A.....	1.5140	0.19	0.125	19	19
Glass B.....	1.5249	0.17	0.111	17	18
Glass C.....	1.5281	0.14	0.092	14	14

t/N_D = equivalent optical path in air.
u = Below limit of correction collar.
† = Surface wavy and irregular.
* = Inhomogeneous optically, varies from region to region.

Lucite was not found to be a satisfactory mounting medium as it faded stained preparations (Richards and Smith, 1938). Styrene (R38, Dow) and Vinylite (National Carbon and Carbide) also caused fading, but Petrex (Hercules) dissolved in methyl salicylate may be useful.[2] O'Brien and Hance (1940) recommended an isobutyl methacrylate film, and a dibutyl phthalate polystyrene mixture was used by Lendrum and Kirkpatrick (1942). Polyvinyl alcohol has been suggested recently by Downs (1943).

Samples of commonly used plastic cover glasses were obtained and their refractive indices (n) measured. From the measured thickness (t) of the material the equivalent air path (t/n) was computed for each and recorded in the table. The plastic covers, and samples of

[2]Personal communication (1939) from J. A. Smith, then of Johns Hopkins University.

glass cover glasses from different manufacturers were mounted with Clarite on a silvered slide of proper thickness to form a Star Test Plate. The silver film has small holes, which, when properly illuminated by the microscope condenser, become point sources. From the position, shape and color of the images of these small points of light the skilled observer can assess the aberrations in the optical system; see Belling (1930).

Two competent observers (I and II) studied the star images and determined the best setting for the correction collar on a Spencer 4 mm. N.A. 0.95 apochromatic objective used with high eyepoint compensating eyepieces. The observations are recorded in the table. The very thin samples of Cellophane and Pliofilm had such short equivalent air paths that it was not possible to correct for the spherical aberration by means of the correction collar. The irregular surfaces also contributed to the blurred images that were seen with the microscope. Such thin materials should never be used on preparations for critical examination.

The other materials listed in the table were thick enough to give optical paths long enough to permit correction for their actual thickness with the adjusting collar of the objective. Plotting the equivalent air paths against the observed correction collar settings showed fairly close agreement for both the plastic substitutes and the glass cover slips with about the same amount of variation in each group. To obtain the best image with the microscope for critical work the correction collar of the objective should be set to the value that corresponds with the actual thickness of the cover slip, just as with glass cover slips.

The Vinylite, and a commercial plastic cover slip presumably Vinylite, judging from its optical constants, developed a brownish coloration of the silver film of the test plate within a few days. Some of this material was also optically inhomogeneous and gave various values for different regions; average values were placed within parentheses in the table. While the refractive index is nearer that of glass, Vinylite may not be satisfactory unless optically homogeneous sheets can be selected.

The dispersion values, showing the relative effect on light of different colors, of the plastics are lower than those for the glass cover slips, but neither observer saw any marked chromatic aberration with the thicker plastic samples. The differences are probably too small to give noticeable color aberration with sheets thin enough to be suitable for microscope cover glasses.

Conclusions

Examination of plastic substitutes for use as cover slips for preparations to be examined with a microscope showed that the thicker ones now on the market are satisfactory when mounted to give a plane surface. Materials optically inhomogeneous, of irregular thickness, that curl or give irregular surfaces, adversely affect the performance of the microscope and should not be used. As the substitutes are softer than glass they must be protected from abrasion. It is recommended that thicknesses of 0.18 mm., and none outside of a range of 0.12 to 0.20 mm. be used. For critical observation with unimmersed objectives of high aperture, the correction collar should be set, for best results, to the position corresponding to the measured thickness of the cover slip, just as would be done with glass cover glasses.

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————. 1941. The use of plastics as a substitute for cover glasses. Science, **93**, 157–8.

RAPID CLEARING OF PIN WORMS (*ENTEROBIUS VERMICULARIS*) FOR CLASS STUDY

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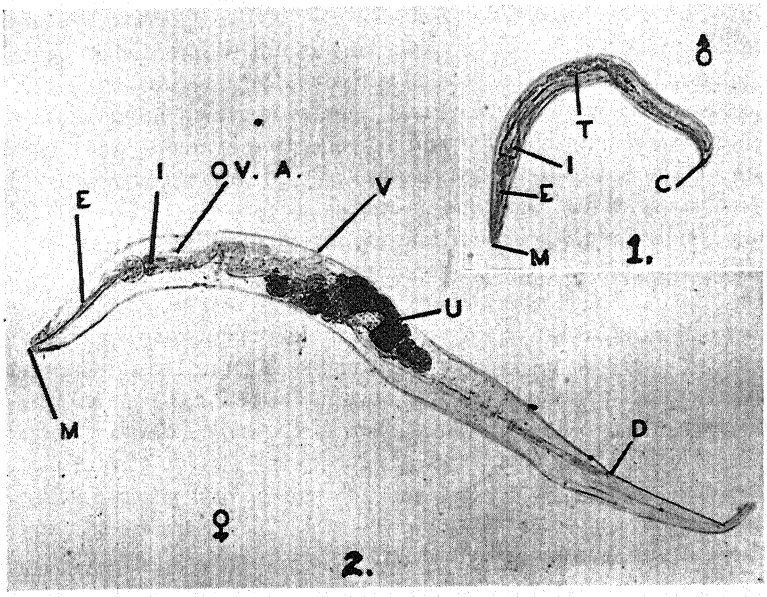
ABSTRACT.—Pin worms can be cleared and differentiated in one operation after formalin fixation and dioxane dehydration by using carbol xylene. The entire procedure, including fixation time, requires less than 3 days. The mounts are permanent and give sufficient detail for class study.



Enterobius vermicularis. 1, male; 2, female.

Explanation of symbols: m, mouth; e, esophagus; i, intestine; t, testis; c, cloaca; ov. a., anterior ovary; v, vagina; u, uterus; d, anus.

Although pin worms are readily available to most laboratories and present no diagnostic problem, they have been found difficult to prepare for class study. The following technic has been found to clear pin worms successfully, leaving good differentiation between internal organs and tissues of the body wall, yielding sufficient detail for class work:

1. Fix in 10% formalin.
2. Wash out formalin in running water, several hours.

3. Dioxane, 3 changes, 1 hour each.
4. Carbol xylene (25 ml. phenol to 75 ml. xylene), 24 hours.
5. Three changes of neutral xylene, a few minutes each.
6. Mount in damar dissolved in xylene and cover.

The worms are handled in a small round bottle. Successive liquids are decanted, and since the worms adhere to the glass, the bottle is thoroughly drained each time by setting it mouth down on filter paper. Individual worms are mounted from the last xylene by using a large caliber medicine dropper.

The body wall is colored a light tan and is translucent. In the anterior end, the prominent digestive organs are a darker tan outlined by a thin black line, while the rest of the digestive system is slightly more yellow. The nerve ring is not differentiated by this technic. When partly distended with ova, the anterior and posterior uteri are dark brown. In gravid females in which huge masses of ova surround the digestive and reproductive systems, the ova are identified in outline only, leaving the colored internal organs visible. Occasionally, a few large ova do not clear, possibly because of their inner, embryonic, lipoidal membrane for chemical protection. This opacity serves to distinguish pre-larval forms from the less developed ova. The anterior ovary is seen as a coiled, striated, yellow tube, often extending as far cephalad as the middle of the esophagus. The posterior ovary is not so easily distinguished from the other organs in that region. Anal and vaginal openings appear as invaginations of the body wall. The digestive system of the male is clearly defined both by color and a thin black line for its full extent. The testis is the same shade of yellow but appears pebbly and is also outlined, Unretouched photomicrographs of male and female specimens. cleared in the manner described, are shown in figures 1 and 2.

NOTES ON TECHNIC

A Modification of the Wirtz Spore-Staining Technic

The modification described here is an outgrowth of attempts to eliminate prolonged heating of dyes on slides.

A heavy suspension, of the organism to be stained, is made in 2–3 drops of water in a 3 × 3/8 inch test tube. An equal quantity of 5% aqueous malachite green is added and the tube is then placed in a boiling water bath for 15–20 minutes.

Smears are made from this stained preparation by spreading a loopful on a clean glass slide, in the usual manner. The smear is allowed to air-dry and when thoroughly dry is fixed by flaming. The prepared smear is decolorized in running tap water for 10 seconds and then counterstained for 1 minute with 0.5% aqueous safranin. The slide is washed again and blotted dry.

This technic stains spores green and vegetative cells red. It has been used on eight species of spore-formers: *Bacillus terminalis*, *Bacillus laterosporus*, *Bacillus alvei*, *Bacillus subtilus*, *Bacillus niger*, *Bacillus vulgatus*, *Bacillus cereus*, and *Bacillus megatherium*, and has been found to give consistently good results in all.

Slides prepared in this manner are much clearer than those made by heating dye directly on the slide. Furthermore, this method has additional advantages: 1) it is easily used, with excellent results, even by inexperienced workers such as undergraduate students; 2) it eliminates the possibility of precipitates, resulting from overheating slides; 3) it consumes much smaller quantities of dye.—Jennie E. Shapiro, *Biology Department, Brooklyn College, Brooklyn, N. Y.*

Anilin Blue as a Counterstain in Cytology

In plant cytology, the usual counterstain used after safranin is fast green F.C.F., or sometimes light green SF yellowish.

When a blue counterstain is desired, anilin blue W.S. is used by some cytologists. Johansen's[1] technic is usually employed, which calls for 2 to 24 hours or even 48 hours in a 1% solution of safranin followed by a saturated solution of the counterstain, the solvent for each dye being equal parts of methyl cellosolve and alcohol with subsequent addition of an equal volume of clove oil to the counterstain. This procedure requires long staining, as well as an unusual solvent.

[1]Johansen, D. A. 1940. Plant Microtechnique. McGraw-Hill Book Co., New York. See p. 82.

The following quick procedure is proposed for the study of chromosomes when using root tips fixed in the CRAF fluid: Remove paraffin from the sections in the usual manner, running down through the alcohols to water. Stain 15 minutes in 1% aqueous safranin 0 and rinse in distilled water. Apply few drops of 1.0% anilin blue W.S. in 95% alcohol[2] for 2 minutes. Dehydrate in absolute alcohol, clear in xylene and mount in balsam.

This gives an excellent picture of cell division and permits microscopic study to be made within 25 minutes. The chromosomes and nuclei are red, the cell walls blue, and the cytoplasm nearly colorless.

Root tips fixed in the specified fluid were found to show sharply stained chromosomes and blue cell walls. The results with Flemming's fluid were not so good, as the contrast between the two dyes was less pronounced, especially in the younger cells at the very tip of the root. With Karpechenko's fixative, good results were obtained by increasing the staining time in alcoholic anilin blue from 2 to 8 minutes.—MARY A. DARROW, *Biological Stain Commission, Geneva, N. Y.*

[2]This dye is sparingly soluble in alcohol, and it is not at all sure that as much as 1.0% can be brought into solution. Experience, however, shows that the results obtained are much inferior if solutions of 0.5% or less are used. The solution should be allowed to stand over night to be sure all the dye possible goes into solution.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

DYES AND THEIR BIOLOGICAL USES

KUHNS, D. M., and FELDMAN, H. A. **Laboratory methods used in determining the value of sulfadiazine as a mass prophylactic agent against meningococcic infections.** *Amer. J. Pub. Health,* 33, 1461–5. 1943.

Surveys for meningococcus carriers must be made before using mass treatment of such carriers with sulfadiazine as a method for controlling epidemics of this disease. A routine which employs dehydrated media, a simple candle jar, and a rapid serological technic is described for facilitating studies for meningococcus carriers. Swabbings of the nasopharynx are streaked onto chocolate agar plates containing para-aminobenzoic acid, which are incubated in candle jars. Suspicious colonies are subcultured to other chocolate agar plates. Pure cultures from the latter are inoculated into fermentation media, and agglutination tests also are run. The fermentation media have been found extremely effective for both meningococci and gonococci, the consistency of the reactions obtained establishing them as valuable adjuncts for the final identification of Neisseria. Reactions may usually be read in 12 hr. or less in the case of meningococci, while the gonococci may require 48 hr. Spinal fluids may be inoculated directly into the media. Because of its agar content, the medium contains so little oxygen that incubation in the candle jar usually is unnecessary. Three sugars are used routinely: dextrose, maltose, and sucrose. All strains of meningococcus ferment dextrose and maltose, but not sucrose. However, the fermentation of these two sugars may be slow in an occasional strain. The basal medium consists of Bacto-tryptose, 20 g.; NaCl, 5 g.; agar, 1 g.; phenol red (0.2% solution), 0.4 ml.; distilled H_2O to 1000 ml. It is adjusted to pH 7.4 and 5 g. of dextrose, maltose or sucrose are added to 1 liter of medium. It should be sterilized for not more than 10 min. at 10 lb. pressure.—*M. W. Jennison.*

ANIMAL MICROTECHNIC

CLAVERDON, M. ARDELLE. **A new fixative for animal tissues.** *Science,* 97, 168. 1943.

The following formula is recommended for a superior general fixative: picric acid, 5 parts; isopropanol, 55 parts; acetone, 30 parts; glacial acetic acid, 5 parts; formaldehyde, (40% by vol., C. P.) 5 parts. Fixation, depending on size and nature of tissues, requires 2 hr. to 4 days. After fixation the tissue is washed in nearly absolute isopropanol 1 to 2 hr., followed by three changes of dioxane, 1 to 3 hr. in each change. Infiltration is begun in ⅓ dioxane, ⅔ paraffin, and completed in three changes of pure paraffin, ½ to 1 hr. for each in vacuum oven. Tissues are sectioned at 7 μ and picric acid is removed from the mounted sections with 1.5% NH_4OH in 95% ethanol prior to staining.—*T. M. McMillion.*

COPLEY, A. L., and ROBB, T. P. **Studies on Platelets. I. The method of Vilarino and Pimentel and a new direct method of counting blood platelets.** *Amer. J. Clin. Path.,* 12, 362–71. 1942.

The authors have tested the method of Vilarino and Pimentel (Klin. Woch., 18, 1252–6, 1939) for counting platelets, and present a simpler, more direct method using a differential staining technic. The procedure recommended is as follows: Draw 1 ml. of a modified Aynaud solution (0.75% NaCl, 3.8% sodium citrate and 3.7% formaldehyde) into a 2 ml. syringe; then by venipuncture draw exactly 1 ml. more of blood into the syringe, and mix the blood with the solution by including a small air bubble and inverting the syringe 5 times. This blood dilution may be kept 24 hr. at room temperature in a stoppered tube before using. To make the count, invert the tube containing the blood dilution 10 times,

and add 0.5 ml. of the mixture to 12.0 ml. of 3.8% sodium citrate solution; mix 0.5 ml. of this second dilution with 0.5 ml. of a 0.2% solution of brilliant cresyl blue in 3.8% sodium citrate and place 1 drop of the final dilution in a counting chamber. The red blood cells usually fade after 10 minutes and the platelets appear as dark blue bodies. The new red blood cells that take the stain are differentiated from the platelets by their size and shape. The method of Vilarino and Pimentel was found to be reliable but much more involved and time-consuming than the present one.—*L. Farber.*

ESSEX, H. E., and DE REZENDE, NILSON. **Observations on injury and repair of peripheral nerves.** *Amer. J. Physiol.*, **140**, 107–14. 1943.

For *in vivo* studies of the repair of the injured posterior auricular nerve of the rabbit, the authors made use of a transparent window, and injected methylene blue into an artery or vein. The effective concentrations of the dye that stained the nerve distinctly and appeared to be tolerated without serious untoward effects were 2.5 to 5% solutions of methylene blue in physiologic saline solution. Concentrations of 15 to 20% caused considerable edema of the whole ear. Staining at the time of injury and subsequently permitted study of the injury and repair of the nerve.—*Elbert C. Cole.*

FULLER, DOROTHEA. **Two new methods of staining vaginal smears.** *J. Lab. & Clin. Med.*, 28, 1475–6. 1943.

The following stains are suggested for staining vaginal secretions in studying the menstrual cycle.

Stain I, for routine smears: Fix smears immediately in 95% alcohol and ether (equal parts) for 5 min., run through graded alcohols (80, 50, 30%) to water. Stain in Ehrlich's hematoxylin diluted one half with distilled water for 2 min. Wash in running water for 4 min. Stain 2 min. in solution A (xylidine ponceau 1 g., distilled water 99 ml., and glacial acetic acid 1 ml., added to an equal volume of acid fuchsin 1 g., distilled water 99 ml. and glacial acetic acid 1 ml.). Rinse in water. Stain 2 min. in solution B (orange G 2 g., phosphomolybdic acid 1 g. and distilled water 100 ml.). Superficial cells stain green, deep cells a slightly darker green, highly cornified cells orange, and less cornified cells pink. Fresh red blood corpuscles stain pink, old ones green.

Stain II, for malignant cells: Fix, run down through alcohols to water, and stain in diluted Ehrlich's hematoxylin as in Stain I. Wash and run through ascending alcohols. Stain 2 min. in a mixture of 95% alcohol saturated with eosin, 11 ml., alcohol saturated with orange G, 35 ml., and alcohol saturated with fast green, 8 ml.). Differentiate with alcohol containing 1% glacial acetic acid for 1 min. with agitation. Rinse in absolute alcohol, then xylene, and mount. This stain does not keep well when mixed. Superficial cells stain green, cornified cells red, and malignant cells are usually red or purple.—*John T. Myers.*

LEBLOND, C. P. **Localization of newly administered iodine in the thyroid gland as indicated by radio-iodine.** *J. Anat.*, **77**, 149–53. 1943.

Guinea pigs and rats were injected with the 8-day isotope I^{131} and dogs and rabbits with isotope I^{130}. In all of the animals except dogs, some ordinary chemical iodine (I^{127}) was added as a carrier. In all cases the iodine was given as a single injection.

At various intervals after injection, the animals were killed and two pieces of thyroid taken from each. One was prepared by ordinary paraffin technic, the other by a modified freezing-drying method after the Altmann-Gersch method, freezing in acetone-dry-ice mixture, drying in a Pyrex test tube connected with a Hyvac pump, and refrigerated at −20° C. Paraffin present in the test tube was melted, thus embedding the tissue, and spreading was done without use of water.

The slides were then put on the sensitive face of medium lantern slide plates and left for several days. Each accumulation of radio-iodine was found to affect the photographic plate. After the plate was fixed and developed, the section was stained with hematoxylin-eosin, or Masson's trichrome stain. The developed plates, or "autographs", show the iodine concentrated in the denser appearing eosinophilic colloid, less abundant in the thinner colloid, and absent outside of the follicles.—*Warren Andrew.*

LILLIE, R. D. **Blood and malaria parasite staining with eosin azure methylene blue methods.** *Amer. J. Pub. Health*, **33**, 948–51. 1943.

This article is a brief discussion of some of the factors involved in the manufacture of satisfactory, reproducible Giemsa and Wright stains for blood and parasite work. Spectrographic analysis and control is essential after standardization of the chemical procedures. Quite satisfactory Giemsa and Wright stains are now available. The absorption spectra correlate well with the staining capacity of the dye mixtures. Stains of the Wright type should have an initial absorption maximum between 650 and 660 mμ.—*M. W. Jennison.*

LILLIE, R. D. **Some experiments on the Romanovsky staining of blood films.** *J. Lab. & Clin. Med.*, **28**, 1872–5. 1943.

Wright stain solutions, made up in methanol in the usual concentration of 125 mg. per 100 ml., will give blood and parasite stains comparable to Giemsa stain in color effects, if the dilutions with buffered water are increased from 1:2 to 1:5 or 1:10, and the time increased to 15–30 min. Previous fixation of the films with 80–100% methanol and staining with premixed dilutions of either Wright or Giemsa stain is the preferable procedure. Addition of 5–10% of acetone to Giemsa stain decreases the staining time by about one-half.—*John T. Myers.*

MCMANUS, J. F. A. **A rapid silver stain for nerve-fibers in formol-fixed paraffin sections of the human spinal cord and medulla.** *J. Path. & Bact.*, **55**, 503–5. 1943.

The following method for rapid silver staining of human spinal cord and medulla has the advantage of giving good results with a relatively short period of impregnation: Fix in formol or formol-saline; dehydrate; cut sections 4–6μ thick; fix sections to slide with egg albumin; and bring down to water. Place slides in chloral-hydrate-silver solution at 58° C. for 30–36 min. (chloral hydrate, 1 g.; $AgNO_3$, 20 g. or less; water to 100 ml.) Rinse in several changes of distilled water and reduce in freshly prepared Bodian's solution (hydroquinone, 1 g.; Na_2SO_3, anhyd., 1 g.; distilled water, 100 ml.) for 10 min. Wash in distilled water and treat for 10 min. with 1% aqueous gold chloride containing 4 drops glacial acetic acid per 100 ml. Wash carefully in distilled water. Reduce in 2% aqueous oxalic acid until fibers are visible under the microscope. (The sections are usually gun-metal gray viewed against a white background.) Wash in distilled water and tap water (a few seconds in each). Place in 5% $Na_2S_2O_3$+5 H_2O for 5–10 min. Wash well in tap water. Dehydrate in alcohol, pass through xylene and mount in balsam. Counterstain if desired. For the central nervous system incubate the sections at 58° C. for 20–30 min. in 0.6% NaOH and wash thoroughly before placing the sections in Bodian's solution.—*S. H. Hutner.*

MENDELOFF, J., and BLECHMAN, H. **A combined elastica-trichrome stain for tissues.** *Amer. J. Clin. Path.*, Tech. Sect., **7**, 65. 1943.

Masson's trichrome stain (Amer. J. Path., **4**, 181, 1928) has been combined with Weigert's elastic tissue stain (Mallory, F. P., Pathological Technique. W. B. Saunders, Philadelphia. 1938. See pp. 168–9) in a technic staining elastic tissue blue-black, smooth muscle red, and collagen green. The procedure is as follows: Wash deparaffinized sections thoroughly in water; stain 60 min. in resorcin-fuchsin mixture (no details given); wash rapidly in acid alcohol; dehydrate and differentiate in absolute alcohol until sections are faintly red; pass rapidly through 70% alcohol to water; stain 8 min. in Harris' hematoxylin and immerse 5 min. in water; stain 5 min. in ponceau-acid-fuchsin-orange-G solution (no details given); wash thoroughly in water, and treat 10 min. with 3% phosphotungstic acid solution; wash thoroughly in water, and treat 5 min. with light green solution (no details given); followed by 3 min. in 1% acetic acid; dehydrate, clear, and mount in gum damar.—*L. Farber.*

WORLEY, LEONARD G., and WORLEY, ELIZABETH K. **Studies of supravitally stained Golgi apparatus. I. Its cycle in the tectibranch mollusc, Navanax inermis (Cooper).** *J. Morph.*, **73**, 365–99. 1943.

Eggs of this mollusc were immersed in dilute methylene blue (National Aniline) in sea water, first at a dilution of about 1:1,000,000, then in one of 1:750,000, later

in 1:500,000, and so on, thus gradually, over a period of 3 or 4 hours, bringing them into a 1:100,000 dilution. These operations were carried on in subdued light, and the embryos subsequently kept in semi-darkness to avoid possible photodynamic effects. When properly employed, the methylene blue stained the Golgi elements of mollusc eggs dark blue, the mitochondria pale blue-green or not at all, while the fatty and albuminous yolk appeared pale yellow-green. The methylene blue was not toxic in the dilutions employed.—*Elbert C. Cole*

PLANT MICROTECHNIC

ARMITAGE, F. D. **Further uses for chlorazol black E and a new stain for botanical sections.** *J. Roy. Micr. Soc.*, **63**, 14–19. 1943.

To a mixture of phenol crystals, 2 parts by weight, lactic acid, 2 parts by weight, glycerin, 1 part by weight, water, 2 parts by weight, sufficient chlorazol black E is added to give the solution the appearance of India ink. After this has stood for a few days, fungal hyphae and sporangia gradually take up the dye and are easily differentiated into their component parts.

For permanent mounts, the fungus, on Czapek agar, is fixed by flooding the culture dish with 70% alcohol. After at least 3 hr. a few ml. of a saturated solution of chlorazol black E in 70% alcohol are added. When the staining is sufficient, the stain is poured off and the culture is dehydrated with alcohol. A portion is cut out and mounted on a slide in a generous amount of euparal. A cover slip is added. Alternatively, the specimen may be dehydrated in dioxane and mounted in a mixture of dioxane, gum sandarac, and camsal.

To stain paper fibers, about 0.5 g. of paper is boiled 1 min. in saturated aqueous chlorazol black E. The excess dye is removed by running water. The fibers are disintegrated by forming a pellet which is rubbed between finger and thumb; then it is boiled in a tube of distilled water and shaken vigorously. A small portion is pipetted on to a clean slide, which is dried on a hot plate at 70° C. Canada balsam or euparal is used as mountant. A better but slower method begins by disintegrating and boiling the fibers as before, then transferring them to a slide where they remain adherent. They are stained by chlorazol black E in 70% alcohol for 30 min. or longer, then dehydrated and mounted in balsam or euparal. Samples of heavily coated paper must be boiled in 5% aqueous KOH, then thoroughly washed before staining.

Differential staining of sections of plant parts is obtained by the following procedure:

Sections of material fresh from the field are placed in 70% alcohol or in a mixture of 5 ml. 40% formaldehyde, 7 ml. glacial acetic, and 90 ml. 70% alcohol. After at least 15 min. (storage in the fixative may be continued several days), the sections are brought down to water and left at least 8 hr. in a mixture of equal parts of 6% $MgSO_4$ and saturated aqueous chlorazol azurine G 200, after heating the mixture to 80° C. and stirring it well. The mixture remains useful for 2 or 3 days after its preparation. After staining, sections are passed quickly through the alcohols to absolute and are mounted in euparal.

Satisfactory staining of sections of herbarium material has been obtained by soaking or boiling in water, then passing them directly into the mixture of $MgSO_4$ and chlorazol azurine G 200.—*C. E. Allen.*

SHEFFIELD, F. M. L. **Value of phloem necrosis in the diagnosis of potato leaf-roll.** *Ann. Appl. Biol.*, **30**, 131–6. 1943.

Potato leaf-roll is a virus disease whose diagnosis in the field is not easy. In affected plants a necrosis of the primary phloem in the bicollateral bundles occurs which is distinct from any abnormality produced by any other cause. The following technic is suggested for demonstrating phloem necrosis: take a piece from the base of a main stem including about six nodes; trim off leaves and branches; cut stem into pieces of suitable length for hand sectioning; place pieces in 50% alcohol; cut sections by hand through nodes; place sections in phloroglucinol solution (1% in 50% alcohol) on a slide; drain after 1 min.; place in 50% HCl for 1 min.; drain; mount in water and examine at magnification of about × 100. In the healthy plant, most of the phloem will be colorless

but fibers may be pink or red; in the infected plant, some primary phloem strands will be yellowish red. If leaf-roll virus is present, some of the six nodes will show phloem necrosis.—*H. P. Riley.*

MICROÖRGANISMS

BERNHARDT, ERNEST. **A simplified method for staining fungi in skin and nails.** *Arch. Dermat. & Syph.*, **48**, 533–5. 1943.

The author recommends the following procedure: Place on a slide a small particle of the material (not exceeding $2 \times 2 \times 0.2$ mm.). Macerate in 10% KOH. Cover. Remove excess alkali by firm pressure under blotting paper. (Maceration is certainly incomplete if the specimen tends to slip out or if air invades the covered space.) Flood the specimen with a drop of cotton-blue-lactophenol and heat it gently, blotting off excess stain from the opposite rim. Then press it firmly under blotting paper. Microscopic examination shows the objects on a slightly bluish background. Flooding the specimen with clear lactophenol removes the last traces of excess stain. Lactophenol is composed of 1 part each of phenol crystals, lactic acid syrup and water and 2 parts glycerin. The staining solution is made up by adding 0.5% of cotton blue Poirrer to the lactophenol, dissolving and filtering. Specimens sealed with Noyer's cement did not show appreciable fading after 1 year of storage.—*J. A. Kennedy.*

GOLDSMITH, J. B. **A differential stain for the demonstration of Trichinella larvae in tissue.** *Trans. Amer. Micr. Soc.*, **62**, 327–8. 1943.

Good contrast between muscle and *Trichinella* larvae either encysted or in migration was secured as follows: Fix in Bouin's fluid overnight; rinse in water or 50% alcohol to remove excess fixative but not picric acid color; stain in bulk in Delafield's or Harris' hematoxylin overnight; destain in acid alcohol until all hematoxylin color has been removed from cytoplasm; alkalize in alkaline ($LiCO_3$ saturated) alcohol. Embed in celloidin or paraffin. Results: muscle fibers, yellow with picric acid; nuclei of muscle fibers and cells of cyst walls, purple; larvae of *Trichinella*, bluish to purple.—*Virgene Kavanagh.*

KRAJIAN, A. A. **A new and dependable method for the demonstration of acid-fast organisms in tissue sections.** *Amer. J. Clin. Path., Tech. Sect.*, **7**, 45–8. 1943.

The author has modified the acid-fast stain by changing the type of nuclear stain and decolorizer, and by introducing a differentiating step. The method is applicable to frozen and paraffin sections fixed in the usual solutions. The procedure is as follows: dehydrate frozen sections, 7–10 μ, blot in filter paper, dip once in celloidin and allow to dry on the glass slide; wash in tap water until surface is wet; gently steam 3 min. with carbol-fuchsin; wash in tap water and treat with arsenic acid alcohol (1% arsenic acid in 60% alcohol), with constant agitation of slide until most of the red color is removed; wash in tap water and treat 2 min. with Loeffler's methylene blue; wash in tap water; dehydrate with 3 treatments of anhydrous ethanol or isopropanol; treat at once 2 or 3 times with isocreosote (equal parts of anhydrous ethanol or isopropanol and beechwood creosote), agitating slide constantly for an even differentiation; blot immediately in filter paper; clear in 3 changes of xylene of 1 min. each and mount in gum damar. Paraffin sections are deparaffinated with 2 changes of xylene of 2 min. each and 2 changes of anhydrous ethanol or isopropanol, washed in tap water, and then treated as frozen sections.—*L. Farber.*

PACKER, R. A. **The use of sodium azide (NaN_3) and crystal violet in a selective medium for streptococci and Erysipelothrix.** *J. Bact.*, **46**, 343–9. 1943.

A selective medium using NaN_3 and crystal violet as inhibiting agents is able to eliminate much of the difficulty in culturing streptococci and *Erysipelothrix rhusiopathiae* from contaminated materials. These bacteria can be inhibited selectively by adding varying amounts of these two agents to a basal medium of tryptone broth or agar. Stock solutions of 0.25 g. crystal violet (94% dye content) in 100 ml. distilled water and of 1 g. NaN_3 in 25 ml. distilled water were used to make the dilutions in the broth or agar.—*Virgene Kavanagh.*

PERRIN, T. G. **A stain for Treponema pallidum.** *Amer. J. Clin. Path. Tech. Sect.*, 7, 28. 1943.

The staining procedure recommended is as follows: Prepare smears on slides from exudate obtained by compressing base of chancre or by scraping surface of ulcer; allow to dry in air or fix in flame; stain 2 min. with heat, or 6 min. at room temperature in a mixture of Ziehl's carbol-fuchsin 4 ml., acetic acid 1 ml., commercial formalin 1 ml., and distilled water 10 ml.; wash by gentle agitation in water and dry in air.—*L. Farber.*

YEGIAN, D., and BUDD, V. **Ziehl-Neelsen technique. Staining properties modified by different preparations of basic fuchsin.** *Amer. Rev. Tuberc.*, 48, 54. 1943.

Yegian and Baisden (J. Bact., 44, 667, 1942) have shown that the staining properties of the tubercle bacillus of the acetate and chloride salts of the possible components of basic fuchsin (rosaniline and pararosaniline) are quite different; acetate salts of the dyes give smears with a large percentage of beaded organisms, whereas relatively few beaded bacilli are found with the chloride salts. It was also observed that a final washing of the stained preparation in 95% ethanol gives smears with bacilli solidly stained, regardless of the dye salt used. The present report is a study to determine which salt of fuchsin is most suited for the demonstration of the tubercle bacillus.

Solutions of pararosaniline acetate and chloride and rosaniline chloride (National Aniline and Coleman & Bell) in 95% ethanol were used. Cultures were washed free of media and suspended in distilled water before being stained. A platinum loopful of the aqueous suspension was spread on a slide and dried at room temperature. The dye solution was then flooded on the slide and steamed gently for 5 min. After cooling for 3–5 min. at room temperature, the dye was drained off, the slide thoroughly washed with water, decolorized with 5% HCl in 95% ethanol for 90 sec.; washed with water and counterstained 30 sec. with brilliant green (1% in 0.01% NaOH).

It was found that the composition of the dye markedly affected the appearance of the tubercle bacillus. Saturated solutions of the acetate salts gave largely beaded smears, whereas weaker solutions gave faintly stained smears with few or no beaded forms. The chlorides of the dyes in saturated solutions gave smears with relatively few beaded organisms; 0.3% solutions gave smears with no beaded organisms and all the bacilli were solidly stained and easily recognized.

The authors recommend that 0.3% solutions of the chloride salts be used in the Ziehl-Neelsen staining technic and that the indefinite term "basic fuchsin" be discontinued, giving instead the exact composition of the dye on the label, such as "Rosaniline Chloride".—*L. Farber.*

BY-LAWS
OF
BIOLOGICAL STAIN COMMISSION, INC.[1]

ARTICLE I

NAME, LOCATION, OBJECTS, ETC.

1. The name of this corporation shall be the "BIOLOGICAL STAIN COMMISSION, INC."

2. The purposes for which said corporation is to be formed are:

(a) The standardization of biological stains, that is dyes or products and preparations of dyes used for any and all purposes in laboratory procedures;

(b) To carry on investigations looking towards the perfecting of the supply of biological stains, and towards the development of new uses for them;

(c) To publish scientific data relating to the nature and use of biological stains;

(d) To issue statements of certification to manufacturers which they may use in accordance with and subject to the rules and regulations from time to time adopted by the Board of Trustees of this organization on different batches of stain that have been tested by the Commission;

(e) To carry on investigations in related fields wherever they seem desirable towards bringing about any of the objects of this organization;

(f) This organization shall take over the duties, rights and privileges of the unincorporated organization known as the Commission on Standardization of Biological Stains, and to own and to dispose of such property as is now being held by the latter.

This corporation is not organized for profit, and no part of the net earnings shall inure to the benefit of any member of the corporation or individual.

3. The principal office of this corporation shall be in the City of Geneva, County of Ontario and State of New York.

4. The seal of this corporation shall have inscribed upon it the words, "Biological Stain Commission, Inc. Corporate Seal 1943."

ARTICLE II

MEMBERSHIP

1. The members shall be members of the Commission on the Standardization of Biological Stains at the time of incorporation, together with such new members as may be elected from time to time by the Board of Trustees. Said members shall remain in good standing as long as annual dues, imposed by the Board of Trustees, are paid. Members shall be entitled to reduced rates or other privileges in connection with Commission publications as may be decided upon from time to time by the Board of Trustees. The Board of Trustees may, in its discretion, fix dues for membership.

[1]These by-laws were adopted at a meeting held in Philadelphia, Feb. 21, 1944, to incorporate and to accept a charter granted to the Biological Stain Commission by the University of the State of New York, as a non-profit corporation. The meeting was attended by six members of the Executive Committee of the Commission on Standardization of Biological Stains, which now becomes the Biological Stain Commission, Inc. The following officers were elected: President, H. J. Conn; Vice-president, W. F. Windle; Secretary, Louis Gershenfeld; Treasurer, S. I. Kornhauser. These four officers constitute the Board of Trustees of the new organization, with the following additional trustees: E. V. Cowdry, J. C. Hinsey, R. D. Lillie, W. J. Robbins, J. T. Scanlan, A. E. Severinghaus, W. D. Stovall.

2. Any national scientific society interested in biological stains may request representation on the Commission. If approval is granted by the Board of Trustees, said society may designate a representative to the Commission. This representative is to be known as an accredited delegate member.

3. Every member shall be entitled to one vote at any meeting of the corporation.

4. Members shall have no personal right, title or interest in or to any of the property and assets of this corporation.

Article III

MEETINGS

1. The annual meeting of this corporation for the election of trustees and for the transaction of such other business as shall properly come before it, shall be held in each year at such time and place as may be determined upon from time to time by the president of this corporation.

2. Special meetings of this corporation may be called at any time by the President, or if he cannot function, by the Vice-president, and if the President and Vice-president are unable to function, the duties of such office shall be assumed by the Secretary, and if the latter cannot function by the Treasurer. Special meetings may also be called upon the written demand of three or more trustees.

3. Notice of meetings, written or printed, for every regular or special meeting of this corporation, shall be prepared and mailed to the last known postoffice address of each member not less than ten days before any such meeting, and if for a special meeting, such notice shall state the object or objects of such meeting. No failure or irregularity of notice of the annual meeting shall invalidate the same or any proceeding thereat.

4. Five members shall constitute a quorum of this corporation for the transaction of business at any meeting, but a less number by a majority vote of those present, may adjourn any meeting from time to time, without notice other than by announcement at the meeting until a quorum be present. Any meeting at which a quorum is present may also be adjourned in like manner by a majority of the members present. At any adjourned meeting at which a quorum shall be present, any business may be transacted which might have been transacted at the original meeting.

5. The order of business at the annual meeting, and as far as possible at all meetings, shall be:

(a) Calling of roll.
(b) Proof of due notice of meeting.
(c) Reading and disposing of any unapproved minutes.
(d) Reports of officers and committee.
(e) Election of trustees.
(f) Unfinished business.
(g) New business.
(h) Adjournment.

6. Any member may at any time waive, in writing, any notice required to be given under these By-Laws.

7. The President, if present, shall preside at all meetings of the corporation and Board of Trustees. In his absence the next officer in due order, who may be present, shall preside. For the purposes of these By-Laws the due order of officers shall be as follows: President, Vice-president, Secretary and Treasurer.

8. The record and proceedings of any meeting of this corporation may be ratified by any member by ratification in writing.

9. At all meetings of this corporation all questions, excepting the question of amendment to these By-Laws, and such other questions, the manner of deciding which is established or regulated by statute or by other special provision of these By-Laws, shall be determined by a majority vote of the members present provided, however, that a quorum must be present.

ARTICLE IV

BOARD OF TRUSTEES

1. The governing power and management of all property, affairs and business of this corporation, and all powers of this corporation, except as otherwise provided, shall be vested in a board of eleven trustees. The eight members of the Executive Committee of the Commission on Standardization of Biological Stains at the time of incorporation, shall become the initial members of the Board of Trustees, and these eight members shall hold office until the election of their successors. They shall convene after the incorporation and elect the three additional members to the Board of Trustees, whose term of office shall be for one year. When any of the following organizations, namely:

Society of American Bacteriologists
American Medical Association
American Association of Anatomists
American Association of Pathologists and Bacteriologists
American Chemical Society
American Public Health Association
American Pharmaceutical Association
American Society of Zoologists

appoints a new representative to the Commission, the new representative does not, however, become automatically a member of the Board of Trustees. The former representative of that Society shall continue to serve as a member of the Board of Trustees until the next meeting or the first occasion thereafter when elections are being held. On such occasion the members present shall, by a majority vote, fill the vacancy created. A majority of the Board must always be Society representatives.

2. Meetings of the Board of Trustees shall be held at such times and places as may be decided upon by vote of said Board from time to time.

3. Special meetings of the Board of Trustees may be called at any time by the President and in the event of his being unable to function then by the Vice-president. Any three members of the board may request in writing a "Special Meeting" of the board. Said request is to be addressed to the President.

4. Five members of the Board of Trustees shall constitute a quorum for the transaction of business at any meeting, but a less number, by a majority vote of trustees present, may adjourn any meeting from time to time without any notice other than by announcement at the meeting until a quorum be present. Any meeting at which a quorum is present may also be adjourned in a like manner by a majority of the trustees present. At any adjourned meeting at which a quorum shall be present, any business may be transacted which might have been transacted at the original meeting.

5. The officers of this corporation shall be elected in each year by the Board of Trustees from the membership of said Board at their first meeting after the annual meeting of this corporation and they shall hold office for one year next ensuing, or until their successors shall be duly elected and qualified. The Board of Trustees shall have power at any meeting to fill any vacancies.

6. The order of business at any regular or special meeting of the Board of Trustees shall be:

(a) Reading and disposal of any unapproved minutes.
(b) Reports of officers and committees.
(c) Election of officers.
(d) Unfinished business.
(e) New business.
(f) Adjournment.

7. Proceedings of less than a quorum of the Board of Trustees may be ratified by any or all of said trustees, but said ratification must be in writing.

8. The affairs of this corporation between meetings of its Board of Trustees shall be managed by an Executive Committee of three, consisting of the President, the Secretary and Treasurer of this corporation. In the event of the offices of secretary and treasurer being held by the same person, then the other member of the Executive Committee shall be the Vice-president. The executive committee shall have all the powers of the Board of Trustees when it is not in session.

ARTICLE V

OFFICERS

1. The officers of this corporation shall be President, Vice-president, Treasurer and Secretary. Of these the Treasurer may hold the office of vice-president or of secretary, if such combination of officers seems desirable to the Board.

2. The initial officers of this corporation shall be those holding the same office in the Commission for Standardization of Biological Stains at the time of the incorporation. They shall hold office until resignation or until a successor is appointed.

3. The President shall preside at all meetings of the corporation and at all meetings of the Board of Trustees; shall sign all contracts and other instruments of this corporation requiring his signature, or as he may be authorized from time to time by the corporation or the Board of Trustees; shall make a report to the annual meeting of this corporation and shall perform such other duties as are incident to his office or are properly required of him by the corporation or the Board of Trustees.

4. The Treasurer shall, by virtue of his office and during his tenure thereof, be the custodian of all funds, securities, evidences of indebtedness, moneys and other property belonging to the corporation. In case of the absence or inability of the Treasurer to perform his duties as treasurer of this corporation, or in case of a vacancy, his powers and duties as treasurer of this corporation shall be delegated by the Board of Trustees to any other member of the Board until such time as the Treasurer is able to act and perform his duties, or until his successor is duly elected and qualified to act. The Treasurer shall keep regular books of account; he shall perform all duties incident to his office or that are properly required of him by the corporation or the Board of Trustees; shall make a final report to the annual meeting of this corporation and shall give bond at the expense of the corporation for the faithful performance of his duties in such sum and with such sureties as may be required from time to time by vote of the Board of Trustees.

5. The Secretary shall issue notice of all meetings of the corporation and Board of Trustees and shall keep minutes thereof. He shall have custody and charge of the corporate seal and other corporate books, papers and documents, except as otherwise provided in these By-Laws, or otherwise directed by the Board of Trustees; shall make such reports and perform such other duties as are incident to his office or are properly required of him by the corporation or the Board of Trustees.

6. In case of the absence or disability of any officer of this corporation or for any other reason that may be deemed sufficient by the Board of Trustees, the Board may delegate his powers and duties for the time being to any other officer or to any member of the Board of Trustees.

7. If at any time it becomes desirable to hold a regular or special meeting of the Board of Trustees for the election of any officer and it cannot be arranged promptly, a member or two members of the said Board of Trustees shall be nominated as a candidate or as candidates for the office in question. Within two weeks of said nomination ballots shall be mailed to all members of the Board of Trustees at their last known address, on which shall be printed or written the name of the candidate or candidates nominated by the Board of Trustees, and the blank space for writing in the name of any other member of said Board for which a vote is to be cast instead of for a candidate nominated by said Committee. The polls shall be open from the day of mailing said ballot until ten o'clock P.M. on the day of election, as specified on the ballots. The said day of election shall be at least two weeks after the date of mailing the ballots. The ballots shall be opened by tellers selected by the Board of Trustees, who shall declare the person receiving the largest number of votes cast to be elected. The officer thus elected shall take office immediately.

8. There shall also be an Editor of STAIN TECHNOLOGY, which office may be held by any one of the other officers and whose duties shall be those indicated in Article VII, Section 2. Said Editor shall be elected in the same manner as the officers except that a candidate for the office need not be a member of the Board of Trustees. If not a member of said Board at the time of his election, he shall become one *ex officio* upon election.

ARTICLE VI

FINANCE

1. The moneys and all valuable effects and securities of this corporation shall be deposited in the name of this corporation in such depositories as the Board of Trustees may designate. The Board of Trustees shall have charge of investing the funds of this corporation. No funds, income or moneys of this corporation shall be invested or re-invested in any securities or property except such securities and property as are authorized as legal investments for Savings Banks by the laws of the State of New York at the time said investment is made; provided, however, that any investments or securities turned over to this corporation by the Commission on Standardization of Biological Stains may be retained or reinvested along with any other income received therefrom from time to time in like securities, in the absolute discretion of the Board of Trustees. No investments or reinvestments of the funds or moneys of this corporation shall be made except upon the authority of the vote of the Board of Trustees or its Finance Committee.

2. A Finance Committee shall be elected by the Executive Committee with the President of the corporation a member ex officio. This committee shall have all the powers of the Board of Trustees with respect to handling of investments and other financial matters between meetings of the Board.

3. All checks, drafts and orders for the payment of money shall be signed by any two of the following officers—the President, Vice-president, Treasurer, Secretary; except that special accounts of limited amounts for purposes specified by the executive committee may be established from which checks may be drawn signed by either the President or Treasurer.

4. The Treasurer or President may endorse for collection or deposit all drafts and checks which are in terms made payable to or are intended to be paid to the Commission.

5. The books and accounts of the Treasurer of this corporation shall be audited by a committee to be appointed by the Board of Trustees.

ARTICLE VII

PUBLICATIONS

1. The official publication of this corporation shall be STAIN TECHNOLOGY. The President, with the approval of the Board of Trustees, shall have the right to arrange for the Commission's sponsorship of other publications relating to the subjects in which the Commission is interested.

2. STAIN TECHNOLOGY shall be in charge of the Editor who shall be responsible for the editorial policies and business management of the publication, and shall have the right to appoint an editorial board; he may delegate specific duties in connection with the editing or publishing of STAIN TECHNOLOGY to any member of said board. The editor shall not commit STAIN TECHNOLOGY or the corporation to any financial obligation without the approval of the Executive Committee.

ARTICLE VIII

AMENDMENTS

1. These By-Laws may be amended, repealed or altered, in whole or in part, by a majority vote of the entire membership of this corporation after having first been approved by the Executive Committee.

2. Voting upon amendments shall be by ballot and may be conducted by mail, in which event the proposed amendment, with ballot, shall be mailed to all enrolled members at their last known address not less than six weeks prior to the date upon the ballot vote is to be taken, which date shall be specified in the ballot and the polls shall be open from the day of mailing the proposed amendment and ballot until ten o'clock P.M. of the day set for the taking of the vote. The ballots cast shall be counted by a teller selected by the Executive Committee. The teller shall render a report to the Board of Trustees and declare the amendment either carried or not carried, as the case may be, and a copy of the teller's report shall thereupon immediately be mailed to every enrolled member of the corporation at his last known address.

BIOLOGICAL STAIN COMMISSION
INITIAL MEMBERSHIP (1944)

BOARD OF TRUSTEES

Officers

President, **H. J. Conn,** New York State Agricultural Experiment Station, Geneva, N. Y. (*representing the Society of American Bacteriologists.*)

Vice-President, **W. F. Windle,** Northwestern University Medical School, 303 E. Chicago Ave., Chicago, Ill. (*American Association of Anatomists.*)

Secretary, **Louis Gershenfeld,** Philadelphia College of Pharmacy and Science, Philadelphia, Pa. (*American Pharmaceutical Association.*)

Treasurer, **S. I. Kornhauser,** University of Louisville Medical School, Louisville, Ky. (*American Society of Zoologists.*)

Other Delegate Members

Cowdry, E. V., Washington University School of Medicine, 4580 Scott Ave., St. Louis, Mo. (representing the *American Medical Association.*)

Lillie, R. D., National Institute of Health, Bethesda, Md. (*American Association of Pathologists and Bacteriologists.*)

Scanlan, J. T., Eastern Regional Research Laboratory, Philadelphia (18), Pa. (*American Chemical Society.*)

Stovall, W. D., State Laboratory of Hygiene, Service Memorial Institute, Madison, Wis. (*American Public Health Association.*)

Newly Elected Trustees

Hinsey, J. C., Cornell University Medical College, York Ave. and 69 St., New York, N. Y.

Robbins, W. J., New York Botanical Garden, New York, N. Y.

Severinghaus, A. E., College of Physicians and Surgeons, 630 W. 168 St., New York, N. Y.

Members

Allen, C. E., Biology Bldg., University of Wisconsin, Madison, Wis.

Beams, H. W., Dept. of Zoology, State University of Iowa, Iowa City, Ia.

Beltran, Enrique, Apartado Postal 1079, Mexico, D. F.

Brentzel, Wanda W., State College Station, Fargo, N. D.

Burke, Victor, State College, Pullman, Wash.

Chandler, Clyde, formerly New York Botanical Garden, Bronx Park, New York, N. Y.

Chapman, George H., 604 Fifth Ave., New York, N. Y.

Cole, E. C., Williams College, Williamstown, Mass.

Constable, Francis W., McCloskey General Hopital, Temple, Texas.

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STAIN TECHNOLOGY

VOLUME 19 JULY, 1944 NUMBER 3

STAINING NUCLEI AND CHROMOSOMES IN PROTOZOA

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ABSTRACT.—Technics for free-living forms such as Paramecium and for parasitic forms such as the opalinid ciliates are described.

Paramecium: Fix paramecia in hot Schaudinn's fluid containing 5% of glacial acetic acid for 5–15 minutes. (A hot water bath for maintaining the proper temperature of the fixative is described.) Dehydrate up to 83% alcohol. Mount the specimens on albuminized cover glasses. (A table for mounting animals on cover glasses is described.) Apply a thin layer of collodion to the cover glass to prevent the loss of the specimens during the subsequent handling. Pass through descending grades of alcohol to water. Mordant in 4% iron alum for 24 hours. Stain in 0.5% hematoxylin for 24 hours. Destain in saturated aqueous picric acid. Rinse in tap water, expose to ammonia vapor for a second, and then rinse again in tap water. Wash in running water for 1 hour. Dehydrate. Clear, then mount in damar.

Opalinid Ciliates: Make smears on cover glasses and fix them while wet. If the opalinids are to be subsequently stained in hematoxylin, fix in hot Schaudinn's fluid (containing 5% of glacial acetic acid) for 5–15 minutes. Pass through descending grades of alcohol to water. Mordant in iron alum for 24 hours. Stain in hematoxylin for 24 hours. Destain in saturated aqueous picric acid. For Feulgen reaction, fix in a modified weak Flemming's fluid for 1 hour. Wash in running water for 30 minutes. Hydrolyze. Leave 3 hours in fuchsin decolorized with H_2SO_3 (Feulgen formula). Wash in H_2SO_3, then in running water for 15 minutes. Dehydrate up to 95% alcohol. Counterstain with fast green FCF for 2 minutes. Dehydrate in absolute alcohol. Clear, then mount in damar.

For a number of years the writer has studied the nuclei and chromosomes in certain Protozoa including both free-living and parasitic forms. The following technics of fixation, mounting, staining and

destaining have proved satisfactory for those studied and it is believed that they could be adapted for some other Protozoa as well. It is with this belief and in response to the request of certain protozoologists that the present paper has been written. The writer does not claim much originality in the technics; his wish will be fulfilled if this paper proves helpful.

I. Free-Living Protozoa: Paramecium

In studying the nuclei and chromosomes in *Paramecium*, careful consideration should be given to the choice of material. The size of the chromosomes may vary greatly in different species and even in different varieties of the same species. Species and varieties with larger chromosomes are more favorable for cytological investigation. Even within a given variety different clones may vary greatly in the staining capacity of the micronuclei. Clones with deep-staining micronuclei are preferable.

The following procedure of fixation, dehydration, mounting, staining, and destaining is suggested:

(1) Fix animals in hot Schaudinn's fluid.[1] To maintain the proper temperature of the fixative, use a hot water bath (Fig. 1).[2] First flood the trough with water. Place labelled embryological cups in the trough which is heated to about 62° C. Heat Schaudinn's fluid (containing 5% of glacial acetic acid) in a small flask to 50° C. and pour it into the embryological cups.

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[1]For handling specimens in fixation and subsequent steps before they are mounted on cover glasses, use "embryological cups" (also called "embryological watch glasses") as containers, especially those which are about 40 mm. square and have a spherica concavity 12 mm. deep and 32 mm. wide at its largest diameter.

[2]This consists essentially of a brass plate with four legs. The plate (12 mm. thick, 160 mm. long and 120 mm. wide) is rimmed with brass strips on all sides. These metal strips (soldered to the plate) are 6 mm. thick and extend 6 mm. above the top of the plate, thus forming a trough. A hole (6 mm. in diameter and 68 mm. deep) is bored into one side of the trough, at a point equidistant from each end, for insertion of a thermometer. Each of the four supporting legs (110 mm. long and 12 mm. in diameter) is provided with a knurled nut permitting levelling and adjustment of the height of the trough. The trough can easily accommodate a large petri dish (approximately 95 mm. in diameter), or two small petri dishes (approximately 70 mm. in diameter), or six embryological cups. The temperature of the brass plate can be kept fairly constant with a microburner. The temperature of the fixative in the embryological cups is about 12° C. lower than that of the brass plate.

The present design is a greatly modified form of that designed by the late Dr. Philip B. A. Powers of the University of Pennsylvania. This instrument may be purchased from the Chicago Apparatus Co., 1735–43 North Ashland Avenue, Chicago, Ill., from The Electric Hotpack Co., 1225–29 Cottman Street, Fox Chase, Pa., or from Wm. Boekel and Co., 509–519 Vine Street, Philadelphia, Pa.

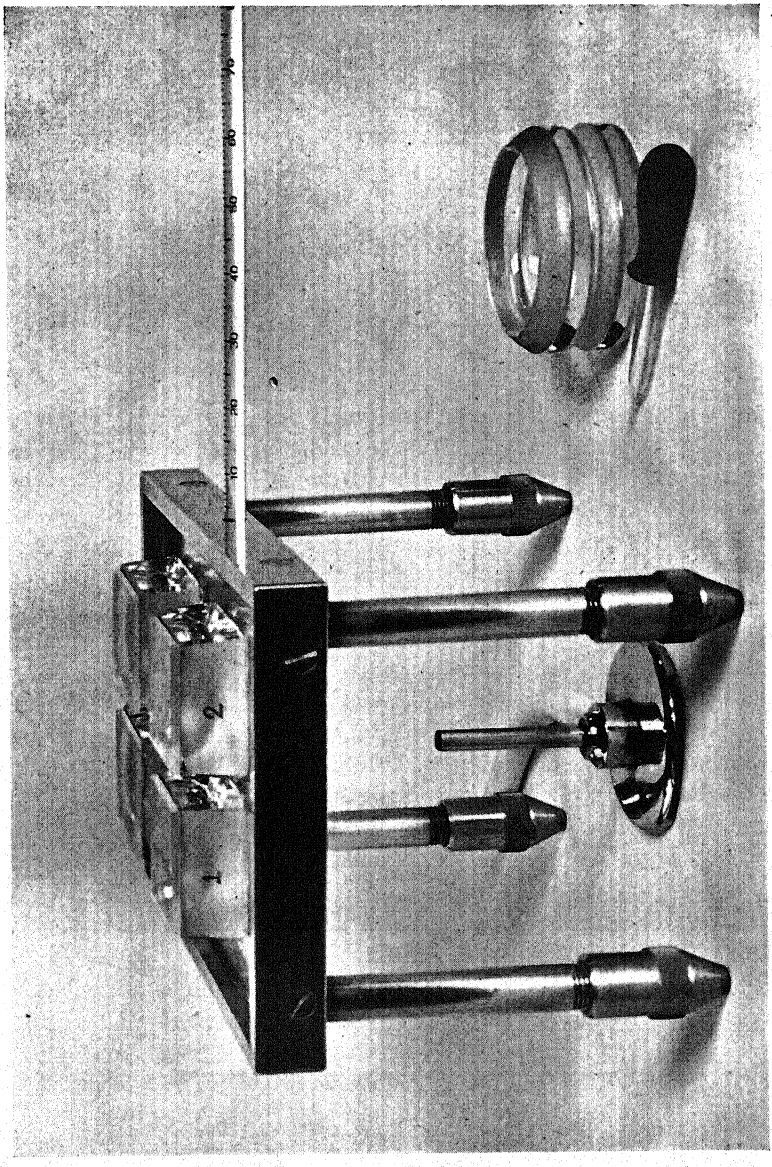

FIG. 1. Hot water bath for maintaining proper temperature of the fixative.

(2) Fix animals for 5–15 minutes. Pour the fixative into a second cup and replace with 50% alcohol. Most of the specimens will stick to the bottom and sides of the original cup. Pick up with a micropipette any loose specimens which go to the second cup with the fixative and return them to the original cup. (This procedure may be omitted if a large number of animals are fixed and if it is unnecessary to study all the fixed animals.) Leave the specimens in 50% alcohol for 10 minutes.

(3) Replace 50% alcohol with 70% alcohol. Leave the specimens in 70% alcohol for 10 minutes or overnight if necessary.

(4) Replace the 70% alcohol with 83% alcohol. Pick up, with a micropipette having a thin-walled tip, the specimens which are stuck to the bottom and sides of the cup and place them in the center of the cup.

(5) Mount the animals on the cover glasses.[3] A table[4] (Fig. 2) for mounting specimens has been designed by the writer. Place a slide on the table which has been made level. Spread Mayer's albumen in a thin layer on a cover glass which is then placed on the slide in such a way that a small portion of it projects over the edge of the slide to facilitate handling (see Fig. 2). With the micropipette pick up as many animals as possible in two or three drops of alcohol. Suck into the pipette sufficient air to produce one or two bubbles in order to break up any clumps of animals that might have been formed in the pipette and drop these animals onto the center of the cover glass immediately. The alcohol, together with the specimens, will spread over the cover glass. Blot off the excess alcohol accumulating on the edges of the cover glass with filter paper. Part of the alcohol on the cover glass will soon evaporate, leaving the specimens in intimate contact with the albumen fixative. Watch the preparation carefully; actual drying must be avoided. Drop one or two drops of 95% alcohol on the cover glass to further harden the fixative. Immediately transfer the cover glass to a petri dish containing 95% alcohol where it remains for 5–10 minutes.

(6) Examine the preparation in the petri dish under a dissecting microscope. Remove with a micropipette specimens that might

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[3]See p. 531 of Wenrich's chapter in McClung's Handbook of Microscopical Technique, *2nd Ed.* Paul B. Hoeber, Inc.

[4]It consists of a brass plate (12 mm. thick, 65 mm. wide and 100 mm. long) with three legs (9 mm. in diameter and 75 mm. in length) each of which is provided with a knurled nut permitting adjustment of table height and making the table level. A circular level is attached to one side of the table as shown in the photograph. The top of the table is painted black. This instrument may be purchased from the Chicago Apparatus Co., 1735–43 North Ashland Avenue, Chicago, Ill.

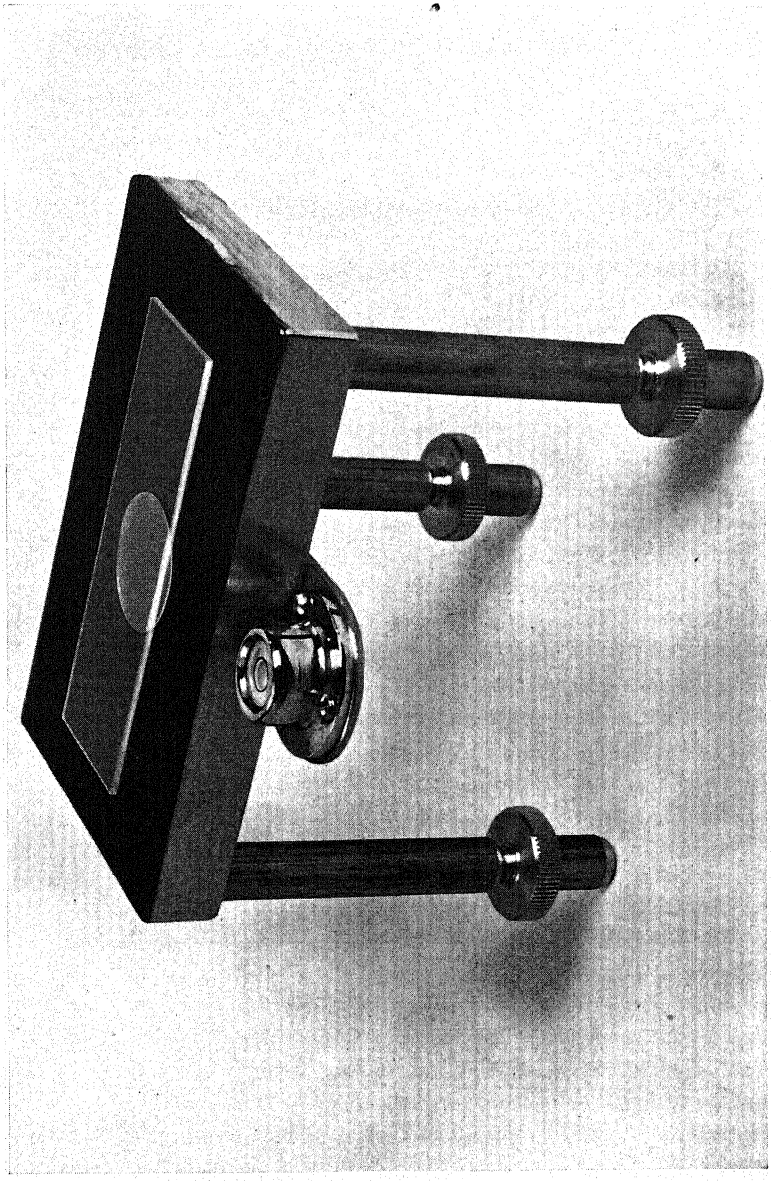

FIG. 2. The table for mounting specimens on cover glasses.

have become loose or clumped together or on top of each other. Also remove specimens which do not lie flat and those on the edges of the cover glass. Return all these specimens to the cup containing 83% alcohol and remount them later. (The above procedure may be omitted if there are many animals mounted and if it is unnecessary to study all the animals.)

(7) Apply a thin layer of collodion to the cover glass to prevent possible loss of specimens during the subsequent handling. This collodion membrane does not interfere with staining and destaining and will be dissolved away during dehydration in absolute alcohol. Use a 1.5% solution of collodion, dissolved in a mixture of equal parts of absolute alcohol and ether. With a pair of forceps in one hand, lift the cover glass, and with the other hand squeeze a few drops of collodion solution from a pipette onto the side of the cover glass containing the specimens. Drain off the excess collodion solution (actual drying of the specimens must be avoided) and place the cover glass immediately in a petri dish containing 83% alcohol, letting it remain there for 5–10 minutes.

(8) Leave for 5–10 minutes in each of the following grades of alcohol: 70%, 60%, 50%, 40%, 30%, 20%, 10% and in water. For carrying out hydration, mordanting, staining, destaining and dehydration of these preparations use either petri dishes or Stender dishes with a staining rack which the writer (1942) has recently described.

(9) Mordant for 24 hours in 4% iron alum.

(10) Wash for 2–3 minutes in running water.

(11) Stain for 24 hours in 0.5% hematoxylin which has been made slightly alkaline by the addition of 3 drops of saturated aqueous solution of Li_2CO_3 to 100 cc. of stain (Galigher, 1934).

(12) Wash for a few minutes in running water.

(13) Destain in a saturated aqueous solution of picric acid. (Tuan, 1930). The length of destaining depends not only upon the size of the animals but also upon the room temperature and the intensity of stain desired. (It is advisable to have several variations of stain intensity especially for the study of the structure of chromosomes.)

Include in a set of preparations one or more cover glasses containing a few specimens as "pilot" preparations. After destaining for some time remove a pilot preparation, rinse in tap water and then in a very dilute solution of NH_4OH (or first rinse in tap water, expose to ammonia vapor for just a second, then rinse again in tap water)[5] and examine under the compound microscope. If the pilot cover glass

[5]See Kidder (1934)

is not sufficiently destained, return it to the picric acid solution. This pilot preparation will, however, be destained at a faster rate than other smears in the same dish which have not been so treated with ammonia water. After some minutes, again take out this pilot preparation, rinse in dilute ammonia water, and reexamine. The procedure is repeated until sufficient destaining is obtained.

Later remove and examine a second pilot cover glass; if it is not sufficiently destained, it should be treated as above. Still later a third pilot cover glass may be taken out for examination. If the specimens in this pilot cover glass are properly destained, remove other preparations in the same dish which contain most of the specimens and treat with dilute ammonia water or with ammonia vapor.

(14) Wash for 1 hour in running water.

(15) Leave for 5–10 minutes in each of the following grades of alcohol: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 83%, 95%, absolute alcohol (one change).

(16) Clear for 10 minutes in cedarwood oil (or in a mixture of oil of cloves and oil of thyme), 10 minutes in xylene (two steps, 5 minutes each).

(17) Mount in damar.

II. Parasitic Protozoa: Opalinids

Opalinids are a group of astomatous parasitic ciliates found chiefly in the rectum of frogs and toads. The relatively large size, the distinct gradations in size and the differences in shape among chromosomes make certain species of opalinids favorable for cytological studies. Species belonging to the genus *Zelleriella* are particularly favorable. The following procedures are recommended:

Make smears on thin cover glasses and fix them while wet. (Good smears can be obtained only by spreading the rectal contents gently, uniformly and thinly over the surface of the cover glass.) If the opalinids are to be subsequently stained in iron hematoxylin, fix them in Schaudinn's fluid containing 5% of glacial acetic acid (at 40°–50° C.) for 5–15 minutes. Transfer the smears to 50% alcohol (remaining for 10 minutes), then to 70% alcohol where they may be left overnight. Pass the smears through the following grades of alcohol: 60%, 50%, 40%, 30%, 20%, 10%, and water (5-10 minutes in each). Mordant in 4% iron alum for 24 hours. Wash in running water for a few minutes. Stain in 0.5% hematoxylin for 24 hours. Destain in saturated aqueous solution of picric acid, and treat with ammonia vapor, etc. as already described for *Paramecium*. (The length of destaining time depends not only upon the size of the animals, but also upon the room temperature and intensity of stain desired; it is best

to have several variations of stain intensity especially for the study of the structure of chromosomes.)

For Feulgen nucleal reaction, a modified weak Flemming's fluid is recommended. To obtain a more or less isotonic fixative, the chemicals to be used are dissolved in normal saline solution except the osmic acid which is dissolved in distilled water in the usual manner. The procedure is as follows: Fix the smears for one hour. Wash in running water for 30 minutes. Hydrolyze in HCl (82.5 cc. concentrated HCl to 500 cc. distilled water) for 5 minutes at room temperature, then for 15 minutes at 60° C. Rinse in cold HCl, then in distilled water. Leave 3 hours in fuchsin decolorized with H_2SO_3 (Feulgen formula). Wash for 1½ minutes in each of 3 dishes of dilute H_2SO_3. Wash in running water for 15 minutes. Leave for 5–10 minutes in each of the following grades of alcohol: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 83%, and 95%. Counterstain with fast green FCF (in 95% alcohol) for 2 minutes. Leave in absolute alcohol for 5 minutes. Clear in cedarwood oil (or in a mixture of oil of cloves and oil of thyme) for 5 minutes, then in xylene (two steps, 5 minutes each). Mount in damar.

For comparison, it is desirable that some smears be fixed in Schaudinn's fluid and stained in hematoxylin while other smears (of material from the same individual host) be fixed in the modified weak Flemming's fluid and stained with Feulgen technic.

A technic for staining the nuclei in avian malaria parasites has also been worked out and will be published elsewhere.

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CHLORAZOL BLACK E IN AQUEOUS ALKALINE SOLUTION

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ABSTRACT.—**Chlorazol black E has a greater solubility in alkaline than in neutral aqueous solution. A method of preparing an alkaline solution (a 1% aqueous solution, heated 5 minutes at 70°C. with 0.2% concentrated ammonia water) for general tissue staining is described, together with notes on its use. The solution was unsatisfactory as a negative stain for bacteria.**

In absolute ethyl alcohol chlorazol black E is slightly soluble. Darrow (1940) recommended a 1% solution in 70% alcohol; Levine and Morrill (1941) found it was not soluble to this extent. The sample of dye used in this work was soluble in 70% alcohol only to the extent of 0.2%. The dye was readily soluble in absolute methyl alcohol. This sample (supplied by Gurr) of chlorazol black E was sparingly soluble in neutral distilled water, approximately 0.3%, although Conn (1943) and Baker (1941) with their samples of dye used a 1% aqueous solution. This difference in the solubility figures given by several workers may be due to variation in the samples used, but the possibility must not be excluded that the workers cited did not actually obtain a 1% solution. Solutions of this dye are so very dense that it is exceptionally difficult to detect undissolved residue.

In aqueous alkaline solutions the dye was found to be very soluble. Solutions up to a strength of 5% were prepared. The solutions obtained using ammonia as the alkali gave the most successful results. The minimum amount of ammonia needed to bring the dye into complete solution was used. When the dilute acid was added drop by drop to an alkaline solution of the dye, it was gradually precipitated. The dye was completely precipitated at pH 5.0.

The following solution was found to be the most practicable:

Stock alkaline solution of chlorazol black E. Add 1 g. of chlorazol black E (Colour Index No. 581) to 25 ml. of distilled water in an Erlenmeyer flask. Mix and then add 0.05 ml. of concentrated ammonia solution (specific gravity 0.880). Warm flask and contents to about 70°C. Keep at this temperature for 5 minutes, stirring gently. Allow to cool. This is the stock solution and it is advisable to keep it in a well-stoppered bottle.

Technic for general stain tissue. The following notes are suggestions. The strength of solution used and time of staining can be varied to suit the worker's requirements.

1. Bring sections to distilled water.
2. Stain 5 to 15 minutes in a mixture of 1 part of stock solution with 3 parts of distilled water.
   (Slight differentiation may be made with 0.1% ammonia in 50% alcohol).
3. Wash in distilled water.
4. Wash in 90% alcohol.
5. Dehydrate in absolute alcohol.
6. Clear in xylene and mount.

The various tissue elements are stained from light gray to dark gray. Connective tissue is well differentiated. Elastic fibers are stained deeply—similar to the effect of staining with an alcoholic solution of the dye as noted by Levine and Morrill (1941). The red blood corpuscles are unstained.

Although the hydrogen ion concentration of the dye solution does not alter the selectivity, it does slightly alter the color of the stained preparation. Thus at an alkaline pH the color tends to be a greenish gray. This can be counteracted if desired by treating with 0.1% acetic acid after stage 3 in the above staining procedure. The color then becomes steel gray.

Chlorazol black E as a negative stain. The stock solution already described is deep black in appearance, very similar to India ink. This solution of chlorazol black E is darker than a similar strength solution of the dye nigrosin and suggested the possible use of this alkaline solution as a background method for unstained bacteria. However, when this solution of chlorazol black E was used as a negative stain it compared unfavorably with the results obtained using nigrosin in similar technics.

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OIL BLUE NA AS A STAIN FOR RUBBER IN SECTIONED OR GROUND PLANT TISSUES[1]

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ABSTRACT.—Oil blue NA (Calco), a stain which colors rubber bright blue, has been used effectively in studying the distribution of rubber in several plant species. Fresh or fixed sections are cut, bleached with Javelle water or NaOCl solution, treated with 9% KOH in 95% ethanol, washed with several changes of water and finally with 95% ethanol, and stained with 0.05% oil blue NA in 70% ethanol. Sections are rinsed in 50% ethanol, placed in 40% glycerin, and mounted in glycerin jelly.

For the detection of changes in the distribution and character of rubber in milled or ground tissues, much the same staining procedure is followed. The stained tissues usually are examined and dissected under a stereoscopic microscope, a procedure which permits rubber to be recognized by both its staining reaction and by a more specific property, elastic elongation.

A microscopic technic is presented whereby it is possible to determine approximately the relative proportion of dispersed and coagulated rubber latex in unstained tissues.

In studies on rubber-bearing plants, histologists (Lloyd, 1911; Hall and Goodspeed, 1919; Spencer, 1939; Artschwager, 1943) have utilized stains (alkanet, Sudan III, Sudan IV) which impart a red, orange, or yellow color to rubber. There is now available, however, a stain, oil blue NA (Calco), which colors rubber clear, bright blue. For some types of histological work, especially with plants having natural yellow and reddish pigments, the blue stain is superior to the red.

Oil blue NA[3] is an oil-soluble stain reported to be 1,4 bis amylamino anthraquinone. That the commercial product may contain a minor impurity is suggested by the fact that under certain condi-

[1]Natural Rubber from Domestic Sources. Paper No. 4.

[2]This is one of four Regional Research Laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

[3]We are indebted to W. H. Peacock of the Calco Chemical Division of the American Cyanamid Company, Bound Brook, New Jersey, for suggesting oil blue NA and making it available to us.

ditions, as when a large excess (0.5%) of the powder is mixed with 50% ethanol, a purple rather than a blue solution is obtained. Washing the powder several times with 50% ethanol selectively removes the more soluble purple component. In the present paper, concentrations of the stain which color rubber blue rather than purple are described.

Like other rubber stains, oil blue NA is not specific for rubber but also stains other plant constituents, such as suberin, cutin, oils, fats, and resins. These materials, however, either can be removed prior to or during staining, or else can be recognized, with the possible exception of resin, by one having a knowledge of plant anatomy and cytology.

The technic to be used for staining rubber in plant tissues depends on the character of the sample and the information sought. The technics presented in this paper have been tested and used effectively at this laboratory in developing methods for the recovery of rubber from plants. We have been interested principally in the distribution and character of rubber in untreated plants and in tracing the changes effected by various chemical and mechanical treatments accessory to recovery or analytical procedures.

Staining of Sections.—The staining technic outlined below has given good results with *Parthenium argentatum* (guayule), *Taraxacum koksaghyz* (Russian dandelion), *Chrysothamnus nauseosus* (rabbit brush), *Actinea Richardsoni* (Colorado rubber plant or pingue), *Cryptostegia grandiflora* and hybrid, and *Landolphia Thollonii.* Sections usually are prepared by sectioning frozen plant material (fixed or unfixed) previously infiltrated under suction with 5% warm gelatin. In some cases, embedding herbaceous and semi-woody tissues in Carbowax 4000 in a manner described by Richards et al. (1942) diminishes the amount of displacement of rubber during sectioning. Treatments (such as paraffin embedding) which dissolve rubber preliminary to sectioning should be avoided.

1. The sections are bleached with 1 ml. of Javelle water or NaOCl solution (containing 5% available chlorine) for 5 minutes at 25° C. in a closed container.
2. To this is added 2 ml. of 9% KOH in 95% ethanol which is permitted to act for 30 to 40 minutes at 25° C. (or for a shorter period at a higher temperature).
3. The mixed solutions are removed from the sections, which are then washed several times with water and finally with 95% ethanol.
4. The sections (in a closed container) are stained for approximately one hour with 0.05% oil blue NA in 70% ethanol.

5. The sections are washed in 50% ethanol for a few seconds, placed in 40% glycerin for a few minutes, and then mounted in glycerin jelly.

Cell or duct inclusions which take the blue stain are usually rubber, since non-rubber substances which take the stain are in a large measure removed by the preliminary treatments. The Javelle water or NaOCl solution destroys protoplasm, clears the cells, and results in a brighter staining of the rubber, whereas the alcoholic KOH dissolves or saponifies suberin, cutin, oils, fats, resins and waxes (Miller, 1938; Rawlins, 1933; Sampson, 1931; Molisch, 1922). When there is any question as to the complete elimination of non-rubber fatty or resinous substances, a portion of the sections should be extracted before staining, some with hot acetone and others with hot benzene. Cell or duct inclusions which take the stain after extraction with acetone and which are removed by extraction with benzene are probably rubber. It should be noted that acetone may dissolve the low molecular weight fraction of rubber[4] and that another fraction may be insoluble in benzene (Memmler, 1934; Stevens, 1943). Sections prepared by the foregoing technic at this laboratory are well preserved and unfaded after eighteen months, during which time several of the slides were subjected repeatedly to microprojection.

Variations in Staining Procedure.—A convenient although perhaps less satisfactory technic than that outlined above for the staining of rubber in plant sections involves the use of a modified Amann's lactophenol. This is prepared by dissolving 0.08 g. of oil blue NA powder in 20 ml. of melted phenol, which is then mixed with 20 ml. of lactic acid, 40 ml. of glycerin, and 20 ml. of water. Extracted or unextracted, bleached or unbleached sections, after being dipped in 25% glycerin, are mounted in the lactophenol solution, which thus serves as a staining, clearing, and mounting medium. The mounts, of course, are temporary.

Another variation permits the staining of rubber simultaneously with the saponification treatment; 0.25% oil blue NA dissolved in the alcoholic KOH will quickly stain rubber, provided a bleaching agent is not present. After being stained, the sections are washed in 50% ethanol, dipped in 40% glycerin, and mounted in glycerin jelly.

Although counterstaining of non-rubber constituents can be accomplished simultaneously with the staining of rubber by mixing safranin, etc., with oil blue NA in step 4, this procedure has been

[4]Unpublished data, Eastern Regional Research Laboratory.

found to offer no advantage in the identification of rubber, since the contrast in brightness between rubber and non-rubber materials is diminished and the background is made more opaque. The bright blue color of the stained rubber can be more readily distinguished against a white background such as is obtained by bleaching the tissues. Counterstaining is desirable only if emphasis is to be placed on non-rubber constituents. In this event the bleaching and saponification treatments should be omitted, since they totally destroy the cytological detail of the non-rubber protoplasmic elements. For cytological studies fixing and bulk staining of tissues preliminary to sectioning may be desirable.

Staining of Ground Tissues.—For staining rubber in ground, milled, or incompletely extracted plant tissues, much the same procedure used for staining sections may be followed. Better results have been obtained, however, by using a weaker staining solution (0.02% oil blue NA in 55% ethanol) for a longer period (18 to 24 hours) and by increasing the initial bleaching period to 30 to 60 minutes, depending upon the nature of the plant material. A Hirsch funnel is convenient for the separation and washing of the tissues from the bleaching and saponifying solution. If permanent records are desired, the stained tissues can be mounted in glycerin jelly. Usually, however, they are examined under a stereoscopic microscope before mounting.

Examination of Ground Tissues.—In a few cases, such as in the examination of *Cryptostegia* leaf chlorenchyma, the compound microscope can be used advantageously for locating rubber in ground tissues. In the majority of cases, however, a wide-field stereoscopic microscope provides the more effective and rapid means. A larger quantity of material can be examined in a shorter time, thus making possible more accurate estimates of the amount of rubber present, and unbroken pieces of tissue can be oriented, dissected, and assayed for rubber.

After the short alcohol wash (step 5), the stained ground tissues are usually placed in water or glycerin in a Syracuse watch glass and examined under a stereoscopic microscope while being probed and manipulated with two needles. Rubber may be recognized not only by its staining reaction but also by a more specific property, namely, its elastic elongation (any dispersed latex rubber will have coagulated during the staining procedure, and thus be elastic). Even small amounts of rubber can be identified by these means, whether it occurs as thin films in individual guayule parenchyma cells or as traces in incompletely extracted kok-saghyz ducts. Indeed,

after some experience with samples of known rubber content, the small amount of rubber remaining in a ground sample that has been chemically analyzed for rubber (Spence and Caldwell, 1935) can be detected and the quantity estimated.

Determining the State of Rubber in Tissues.—It should be pointed out that staining cannot be used in studying all the problems arising in the microscopic examination of plant tissues for rubber. During a staining procedure, chemical and physical alterations are produced, including coagulation of any dispersed rubber latex. Only by direct microscopic observations on untreated living tissues is it possible to estimate the relative proportion of dispersed and coagulated rubber present. No other means is known to us for the approximate determination of this ratio, since chemical methods of of analysis give information only on total rubber and mechanical treatment of tissues causes latex coagulation. The direct microscopic method described below as applied to guayule must be correlated with chemical analyses of the plant and with histological studies of stained sections.

Sections (or ground tissues), cut and kept under water or a latex-stabilizing solution, are dissected immediately under a stereoscopic microscope. When a latex-bearing cell is ruptured, a minute white cloud of latex is seen to issue from it if the latex is in the dispersed condition; if coagulation has occurred, the rubber of an individual cell can be stretched into a thin elastic strand. Dispersed latex can readily be distinguished from coagulated latex even in the same cell, and cells rich in rubber contrast sharply with cells of low rubber content. With surface illumination and dark background, cells containing dispersed latex appear opaque white; as latex coagulation occurs, they change to slightly translucent white, then to light yellow, and finally to brown. Of course, in chlorophyllous tissue, the color changes are modified. As a check upon the amount of coagulated latex remaining in a ruptured and crushed cell, it can be stained and re-examined, the stereoscopic and compound microscopes being used interchangeably. Under the compound microscope, dispersed latex in fresh, unstained tissues is distinguished by its Brownian movement.

After some experience with a given plant species, an histologist following the technic just described is able to make a fairly reliable estimate of both the condition and quantity of rubber even in unstained tissues. Such estimates have been of material value in developing large-scale methods for the recovery of dispersed rubber latex from both guayule and kok-saghyz, since they indicate the

effect of a recovery step on latex coagulation in tissues. The technic has been employed also in investigating the state of rubber in tissues of anatomically unfamiliar rubber-bearing plants. This knowledge is necessary for the intelligent planning of a recovery procedure, and may indicate whether the rubber can best be extracted by bleeding, solvents, retting, crushing, milling, or by some other means.

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A DIFFERENTIAL STAIN FOR RUBBER IN GUAYULE

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ABSTRACT.—**The following schedule, which combines an intense blue stain for rubber with sharply contrasting red counterstains, has been found satisfactory for use in an anatomical study of rubber deposition in guayule: Cut fresh or fixed sections about 50 to 100 μ thick, transfer to 50% ethanol. Extract with acetone 5 minutes, treat with 1% NaOCl 5 minutes, saponify with 10% KOH in 95% ethanol 15 minutes, rinse 3 times with 50% ethanol, stain in oil blue NA (Calco) with safranin and Congo red 30 minutes at 55°C. Rinse in 50% ethanol 2 (or more) times to remove excess stain and mount in Karo syrup.**

The present widespread work on rubber-yielding plants may make the following method, which was developed for guayule, of some general interest. Many stains have been employed to aid in the detection of rubber. These include alkanet (Lloyd, 1911), Sudan III (Hall and Goodspeed, 1919, Artschwager, 1943), and most recently oil blue NA (Calco)[1].

The last appears to be the most satisfactory in giving a sharp, easily distinguished stain. However, none of them is specific for rubber, all stain lipids, waxes, essential oils, resins, and similar compounds as well. It is therefore necessary to remove all of these except rubber, preliminary to staining. The writer makes no claim for originality in the development of a pretreatment for the tissues, or for the use of Calco oil blue. The following staining combination, however, has not been previously described to the writer's knowledge. It has the advantage of staining the prominent anatomical structures as well as rubber. Thus a single slide can show not only the distribution of rubber, but also clearly defines the extent of the cork, cortex, phloem, xylem, pith, cell wall development, and other features, as well.

1. Fix the materials in formalin-propiono-ethanol (Johansen, 1940, p. 41-2) for 24 to 48 hours.

[1]Report of War Activity, May 1, to Oct. 1, 1942, Eastern Regional Research Laboratory. Bureau of Agricultural and Industrial Chemistry. U. S. Department of Agriculture. Unpublished. See also preceding paper by Whittenberger, p. 93.

2. Section at 50-100μ. Collect sections in 50% ethanol.
3. Extract in acetone 5 minutes.
4. Bleach with 1% NaOCl for 5 minutes.
5. Saponify with 10% KOH in 95% ethanol 15 minutes.
6. Rinse 3 times with 50% ethanol.
7. Stain in oil blue NA with safranin O and Congo red for 30 minutes at 55°C. (Formula given below).
8. Rinse sections 2 (or more) times with 50% ethanol.
9. Mount in Karo syrup (Crystal White).

With this procedure rubber is stained blue, lignified tissues and cork orange red (vermilion), cellulose and cytoplasm light red (rose).

Preparation of Solutions

The stock solution of oil blue was a saturated solution of Calco oil blue NA, (No. 77066 Calco Chemical Division, American Cyanamid Company) in 190 ml. of 50% ethanol plus 10 ml. of tertiary butyl alcohol. The solution was obtained by dissolving approximately 0.5 g. of oil blue NA in the tertiary butyl alcohol at room temperature and then adding the ethanol. After shaking a few times the solution was filtered and was then ready for use. The stock solution of safranin O (Johansen, 1940, p. 62) was made with 4 g. safranin O (Cert. No. NS-16, dye content 86%); 200 ml. methyl cellosolve; 100 ml. of 95% ethanol; 100 ml. H_2O; 4 g. sodium acetate; 8 ml. formalin. The Congo red employed carried Cert. No. CQ-2 and had a dye content of 70%.

We have obtained good results by combining the three stains in one solution. A mixture of oil blue NA and safranin stock solutions in proportion of 50 to 1 has been found to be the most satisfactory. To this was added from 0.2 to 0.4% by weight of Congo red. It was difficult to remove excess safranin from our sections by destaining procedures, and so the above combination of stains was selected giving the desired colors without overstaining.

Discussion

Fixation of tissues appears to be desirable as it renders the cell contents less liable to change by the rather drastic treatments preliminary to staining. However, when the gross distribution of rubber is the principal objective, fixation can be omitted.

Sectioning is accomplished with a sliding microtome equipped with a CO_2 freezing attachment. Use of this accessory facilitates rapid mounting and firm holding of materials to be sectioned. Freezing time is minimal if the pieces of material are made as short as

possible. They can be conveniently cut from stems or roots without damage to the tissues by means of an inexpensive power-driven jigsaw. Three to five millimeters was found to be the shortest practicable length to which blocks could be cut. They are mounted for freezing in a 40% solution of gum acacia in distilled water, a preparation whose use permits freezing at a higher temperature than does the frequently recommended sugar-gum solution.

The acetone treatment is included to insure that resins, which stain with oil blue NA, are removed. It is possible that this step is not necessary since the resins of guayule are soluble in alcohol. Evidence has been obtained which indicates that the resins are completely removed by the various alcoholic solutions through which sections pass. However, the acetone treatment is recommended as an added precaution. In addition, it aids in the extraction of the chlorophyll pigments which tend to mask the rubber stain in the outer layers of the cortex.

The NaOCl acts as a bleach, principally of the chlorophyll pigments. This treatment and the acetone extraction may be omitted in many cases, if their clearing effect is not required.

Saponification of sections with 10% KOH in 95% ethanol for 15 minutes removes the suberin from the cell walls of the cork. Occasionally material may be encountered which requires longer treatment. If this treatment is omitted, however, the cork stains purple, apparently taking up both oil blue and safranin.

After saponification, the cork stains red, taking up only safranin. The cuticle of the trichomes, however, is much more resistant to saponification. Several hours of treatment with hot KOH is necessary in order to remove all the blue-staining material from these hairs of the leaves and young stems.

Thirty minutes in oil blue with safranin and Congo red at 55°C. was necessary in order that the tissues be thoroughly stained. No ill effects have been noticed when it was necessary to leave sections in stain overnight.

The Congo red or safranin or both may be omitted with the sections in which it is desired to study only the deposition of the rubber. In older tissues which have well developed cell walls in the parenchyma, or in thicker sections (over 70μ), Congo red tends to obscure the rubber as it stains the walls of the rubber-containing cells. However, when it is desired to study the development of rubber bearing tissue (parenchyma) as well as rubber deposition, the addition of Congo red has definite value especially if the sections are cut below 70μ. Safranin stains lignified tissues and cork in

the method outlined above. As these are the principal non-rubber-bearing tissues it is advantageous to have them stained a contrasting color. When employed with oil blue NA, the counterstains enable the simultaneous study or demonstration of the anatomical features of a section and its rubber distribution.

Karo syrup (Crystal White) has been found to be a satisfactory mounting medium. Sections can be placed in it directly from 50% ethanol. It hardens quickly on a warming plate, and becomes as firm as balsam or clarite.

In general, the schedule outlined above is flexible and may be varied with the material being studied and the requirements and facilities of the individual laboratory.

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CONTROL OF THE FERRIC ION CONCENTRATION IN IRON-ACETO-CARMINE STAINING[1]

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ABSTRACT.—The Fe^{+++} concentration is controlled by adjusting the $FeCl_3$ normality of the iron-aceto-carmine staining solutions. Two stock mordant solutions are prepared by dissolving ferric chloride ($FeCl_3 \cdot 6H_2O$; m. w. = 270.31) in 45% glacial acetic acid, the normality of one being N/1, and of the other N/10. By combining aceto-carmine (preferably prepared from Merck's carmine No. 40 N. F., or from the Coleman and Bell product) and one or the other of the stock mordant solutions, a series of iron-aceto-carmine solutions is made up, each solution being of different normality for $FeCl_3$, depending on the proportions combined. Trial series: N/50, N/100, N/500, N/1000 and 0 N.

Tissue (spermatogenic) from one specimen is fixed in Carnoy-2, divided equally among the five iron-aceto-carmine solutions for staining, then squashed, dehydrated and mounted as usual. Subsequently the trial series may be retained or adjusted. Advantages of the method: 1) discloses quickly the optimum stain for a particular tissue type; 2) automatically gives an optimum stain to cells in different maturational stages; 3) results are reproducible in subsequent operations.

Tables and equations are provided for a number of other normalities and quantities of stain.

It is usually unnecessary to determine quantitatively the amount of iron, or iron compound, to be added to aceto-carmine solutions; the extent to which this stain has been successfully used in smear preparations indicates in itself that for a majority of uses qualitative addition of the mordant is satisfactory. In some instances, however, more precise methods prove to be exceedingly helpful. Such an instance is encountered in the preparation of mammalian hybrid tissues for chromosome determinations, in which the following points are to be considered: (1) the number of hybrid individuals obtained is often small, and all tissue must be utilized to the fullest advantage; (2) since there is little or no tissue which can be spared for the usual type of staining experiment, the method must disclose

[1]Contribution from the Zoological Laboratories, University of Michigan, Ann Arbor, Mich.

an optimum staining solution at first trial; (3) cells in different stages of spermatogenesis have different stain affinities, and it is a decided advantage to have each one of the various stages stained in as satisfactory a manner as is possible; (4) the method must give reproducible results in subsequent applications. In attempting to meet these requirements, the following method has been devised.

Since mordant strength is dependent upon the ferric ion concentration, this concentration is controlled by adjusting the normality of the staining solution for $FeCl_3$. Stock mordant solutions are prepared by adding ferric chloride ($FeCl_3 \cdot 6H_2O$) to 45% glacial

TABLE 1.—GRAMS OF $FeCl_3 \cdot 6H_2O$ IN SOLUTIONS OF DIFFERENT NORMALITY AND VOLUME

To determine $FeCl_3$, multiply $FeCl_3 \cdot 6H_2O$ by 0.6; to determine Fe^{+++}, multiply $FeCl_3 \cdot 6H_2O$ by 0.205.

Normality	Quantity of solution, V_2			Dilution ratio* from	
	10 ml.	100 ml.	1000 ml.	N/1	N/10
N/1	0.901	9.010	90.103	1: 0:1	———
N/10	0.090	0.901	9.010	1: 9:10	———
N/20	0.045	0.450	4.505	1: 19:20	———
N/30	0.030	0.300	3.003	1: 29:30	———
N/40	0.022	0.225	2.252	1: 39:40	———
N/50	0.018	0.180	1.802	1: 49:50	———
N/60	0.015	0.150	1.501	1: 59:66	———
N/70	0.013	0.129	1.287	1: 69:70	———
N/80	0.011	0.113	1.126	1: 79:80	———
N/90	0.010	0.100	1.001	1: 89:90	———
N/100	0.009	0.090	0.901	1: 99:100	1: 9:10
N/200	0.004	0.045	0.450	1:199:200	1:19:20
N/300	0.003	0.030	0.300	1:299:300	1:29:30
N/400	0.002	0.022	0.225	1:399:400	1:39:40
N/500	0.0018	0.018	0.180	1:499:500	1:49:50
N/600	0.0015	0.015	0.150	1:599:600	1:59:60
N/700	0.0013	0.013	0.129	1:699:700	1:69:70
N/800	0.0011	0.011	0.113	1:799:800	1:79:80
N/900	0.0010	0.010	0.100	1:899:900	1:89:90
N/1000	0.0009	0.009	0.090	1:999:1000	1:99:100

*Dilution ratio given as $V_1:(V_2-V_1):V_2$.

acetic acid. Two useful stock solutions, expressed in terms of normality for $FeCl_3$, were found to be N/1 and N/10. The N/1 solution is prepared by adding to 9.01 g. of $FeCl_3 \cdot 6H_2O$ (m. w. = 270.3) an amount of 45% glacial acetic acid to make 100 ml. of finished solution; the N/10 solution is made by dilution from the N/1. These, as well as other concentrations and amounts, may be made from the data of Table 1.

Immediately before use, staining solutions are prepared by combining measured proportions of aceto-carmine (prepared from

carmine, Merck's No. 40 N. F. or the Coleman & Bell product, by any of the usual procedures[2]) and stock mordant solutions to give a series of stains, each of different normality. These normalities may be calculated as follows:

let V_1 = volume of mordant solution,
N_1 = normality of mordant solution,
V_2 = volume of iron-aceto-carmine solution,
N_2 = normality of iron-aceto-carmine solution,
(V_2-V_1) = volume of aceto-carmine solution, and
N_0 = normality of aceto-carmine solution for $FeCl_3 = 0$;

then $V_1N_1 + (V_2-V_1)N_0 = V_2N_2$,

but since the normality (N_0) of aceto carmine for $FeCl_3 = 0$, $(V_2-V_1)N_0$ may be omitted from calculations. Then

$$V_1N_1 = V_2N_2 \quad , \quad \text{or} \quad V_1 : V_2 :: N_2 : N_1.$$

Iron-aceto-carmine of any normality and in any amount may be prepared by these formulas.

TABLE 2.—PREPARATION OF IRON-ACETO-CARMINE SOLUTION

Add aceto-carmine to $FeCl_3$ solution of indicated normality (N_1) and volume (V_1) to make iron-aceto-carmine solution according to the normality (N_2) and volume (V_2) required.

$$V_2 = (N_1V_1)/N_2$$

N_1		V_1						Dilution ratio, $V_1:V_2$
N/1	N/10	0.05	0.1	0.5	1.0	5.0	10.0	
N/10	N/100	0.5	1	5	10	50	100	1:10
N/20	N/200	1.0	2	10	20	100	200	1:20
N/30	N/300	1.5	3	15	30	150	300	1:30
N/40	N/400	2.0	4	20	40	200	400	1:40
N/50	N/500	2.5	5	25	50	250	500	1:50
N/60	N/600	3.0	6	30	60	300	600	1:60
N/70	N/700	3.5	7	35	70	350	700	1:70
N/80	N/800	4.0	8	40	80	400	800	1:80
N/90	N/900	4.5	9	45	90	450	900	1:90
N/100	N/1000	5.0	10	50	100	500	1000	1:100
N_2		V_2						

Table 2 summarizes a convenient procedure for the preparation of larger volumes of stain. Here the measurements involved are N_1, V_1 and V_2. V_2 volumes not given directly in Table 2 are easily computed since the value of V_1 and the corresponding values of V_2 in the same vertical column may each be either multiplied or divided

[2]The brands of carmine named proved to give the best results out of several tested; the author, however, does not consider other brands necessarily unsatisfactory.

by the same number, without altering the normality of the iron-aceto-carmine. For example, to make 30 ml. of N/100 iron-aceto-carmine from aceto-carmine and N/10 stock mordant,

$$3(1.0\ V_1{:}10\ V_2) = 3\ V_1{:}30\ V_2.$$

Then, to 3 ml. of N/10 stock mordant is added aceto-carmine to make a finished volume of 30 ml. of iron-aceto-carmine, the normality of which will be N/100. If desired, any given case may be computed from the equation on which Table 2 is based—$V_2 = (N_1V_1)/N_2$.

Table 3 summarizes a more applicable method for the preparation of small quantities of stain, in which V_1 and $(V_2–V_1)$ are measured by pipettes. Measurements involved here are N_1, V_1 and $(V_2–V_1)$.

TABLE 3.—PREPARATION OF IRON-ACETO-CARMINE SOLUTION

Add the volume of aceto-carmine $(V_2–V_1)$ indicated to the volume (V_1) and normality (N_2) of $FeCl_3$ solution indicated, to make iron-aceto-carmine solution according to the normality (N_2) and volume (V_2) required.

$V_1 = (N_2V_2)/N_1$

N_1		V_2					Dilution ratio, $V_1{:}(V_2–V_1)$
N/1	N/10	(1) 0.8–1.4	(5) 4.5–5	(10) 9.6–10	(20) 19.2–20	(50) 45–50	
N/10	N/100	0.1 +0.9	0.50 +4.50	1.00 +9.00	2.00 +18.00	5.0 +45.0	1:9
N/20	N/200	0.05 +0.95	0.25 +4.75	0.50 +9.50	1.00 +19.00	2.5 +47.5	1:19
N/30	N/300	0.04 +1.16	0.16 +4.64	0.33 +9.57	0.66 +19.14	1.6 +46.4	1:29
N/40	N/400	0.03 +1.17	0.12 +4.68	0.25 +9.75	0.50 +19.50	1.2 +46.8	1:39
N/50	N/500	0.02 +0.98	0.10 +4.90	0.20 +9.80	0.40 +19.60	1.0 +49.0	1:49
N/60	N/600	0.02 +1.18	0.08 +4.72	0.16 +9.44	0.32 +18.88	0.8 +47.2	1:59
N/70	N/700	0.02 +1.38	0.07 +4.83	0.14 +9.66	0.28 +19.32	0.7 +48.3	1:69
N/80	N/800	0.01 +0.79	0.06 +4.74	0.12 +9.48	0.24 +18.96	0.6 +47.4	1:79
N/90	N/900	0.01 +0.89	0.05 +4.45	0.11 +9.79	0.22 +19.58	0.5 +44.5	1:89
N/100	N/1000	0.01 +0.99	0.05 +4.95	0.10 +9.90	0.20 +19.80	0.5 +49.5	1:99
N_2		$V_1+(V_2–V_1)$					

If desired, the proportions for untabulated V_2 volumes may be readily determined, since the values of V_1 and of (V_2-V_1), for the same value of N_2, may each be either multiplied or divided by the same number, without altering the normality of the stain solution. It will be noted that in this table V_2 volumes are approximated—(10) ranges from 9.6 to 10 — since in this method of preparation the exactness of the V_2 volumes is of but secondary importance. The point of primary importance is the ratio $V_1 : (V_2-V_1)$. V_2 is distributed over a small range in order that V_1 and (V_2-V_1) need be measured to only the second decimal place, rather than to an inaccurate third or fourth, and the correct dilution ratio still retained. Again, any given case may be computed from the equation on which Table 3 is based — $V_1 = (N_2V_2)/N_1$.

From the tabulated data a series of iron-aceto-carmine solutions of varying normality is prepared. Which normalities to use must of course be determined for various different types of tissue, and one member of the series should serve as a control, i.e. should be an aceto-carmine solution without mordant. A trial series which has been found useful is N/50, N/100, N/500, N/1000 and 0 N. Tissue previously fixed in Carnoy-2 (20 to 30 minutes) is divided among the five solutions for staining (30 to 60 minutes); after this, squashing, dehydrating and mounting are carried out according to standard procedure.

When staining tissue taken from one specimen, at least one stain of the series may be expected to fall near the optimum for the tissue. Then, as is desired, an adjusted and sometimes smaller series may be subsequently used; or only one member of a series may be used if this is deemed satisfactory. The continued use of an adjusted series for each individual specimen is particularly valuable in staining gametogenic tissues, since cells in the various maturational stages are each automatically given an optimum stain.

Precautions. Ferric chloride ($FeCl_3 \cdot 6H_2O$) is markedly hygroscopic, and care must be taken to prevent absorption of water in the stock supply and in samples being weighed. The practice of using stock mordant solutions is a convenience not only in adjusting the normality of the staining solutions, but also in reducing the possibility of absorption of water by the ferric chloride.

In preparing the staining solution, aceto-carmine is necessarily diluted somewhat by addition of the mordant solution, since the latter contains no carmine. To minimize this dilution, the tables are so constructed that the volume ratio of mordant solution to aceto-carmine is never greater than 1:9. Consequently small

volumes, such as those of Table 3, require the use of pipettes for accurate measurement.

Only fresh iron-aceto-carmine solutions should be used for staining.

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IMPROVING SMEAR TECHNICS BY THE USE OF ENZYMES

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ABSTRACT.—A method for securing spreading of large meiotic chromosomes is described. It consists in treating a piece of fixed anther in a 1% solution of Clarase, a proprietary enzyme complex, or in an extract prepared by grinding the contents of flask cultures of certain fungi (Aspergillus niger, Chaetomium globosum, Metarrhizium sp.) with quartz sand in a mortar containing 10 ml. of a sodium acetate buffer, pH 5.0. The fixed anthers are thoroughly washed in H_2O prior to the enzyme treatment. Length of treatment may vary from as little as 10 minutes to several hours. The usual aceto-carmine or propionic-carmine smear technic may then be used. The treatment destroys some of the elasticity of the cytoplasm so that the chromosomes remain spread out when light pressure is exerted on the cover slip.

The development and improvement of the smear method for preparation of cytological specimens has made possible much of the recent critical work on chromosomes. In some plants with large chromosomes, however, there still exists some difficulty because of chromosome clumping. This is especially true where polyploids are concerned and when chromosome associations at the first meiotic metaphase are being studied. This difficulty was encountered with a colchicine-induced tetraploid of *Lilium longiflorum* Thunb. produced at the Plant Industry Station, Beltsville, Maryland.[1] In order to overcome this, a number of treatments designed to digest the cell wall and destroy the gel properties of the cytoplasm were tried. A preliminary paper has already appeared.[2] In the present paper the technics and results are given in greater detail.

The plants used included, in addition to the tetraploid *Lilium longiflorum*, the triploid *L. tigrinum* Ker Gawler, and several dip-

[1]Emsweller, S. L., and Lumsden, D. V. Polyploidy in the Easter lily. Proc. Amer. Soc. Hort. Sci., 42, 593–96. 1943.

[2]STUART, NEIL W., and EMSWELLER, S. L. 1943. Use of enzymes to improve cytological techniques. Science, 98, 569–70.

loid forms of *L. longiflorum*. Buds were collected in which the pollen mother cells were mostly at the first meiotic metaphase. After removing the perianth segments, the buds were fixed in a mixture of absolute alcohol (3 vol.) and glacial acetic acid (1 vol.). The next day they were run through 95% alcohol to 70% where they were kept until needed. The best results were secured when the buds were used a few days after fixation. The anthers were cut into 1 to 2 mm. pieces, thus making it possible to give a series of different treatments to pollen mother cells from the same anther. Following treatment, the piece of anther was placed on a slide and the pollen mother cells dragged out of each locule with a bent needle. This was carried out while under observation on a binocular dissection microscope. In this way pollen mother cells only are secured on the slide. The finest slides were secured when few cells were used, since they could be scattered and more easily flattened. All slides were stained with aceto-carmine.

For the first tests only inorganic chemical treatments were used. They were: copper oxide ammonia (Schweitzer's reagent), a cellulose solvent; 1% ammonium oxalate, a pectic solvent; and 10% alcoholic KOH, a suberin solvent. All the slides from treated material were inferior to the untreated checks.

These unsatisfactory results led us to try a biological approach to the problem by means of enzymes. Three preparations were used: Malt diastase; Polyzime "P", used in desizing fabrics; and Clarase,[3] a proprietary enzyme complex. The fixed anthers were run through the lower alcohols and thoroughly washed in water. Each anther was then cut into four small pieces so that a portion of each could be placed in the three enzyme preparations, and one left in water. Various periods of immersion in the 1% enzyme solutions were tried, in all instances following a presoaking interval in water. None of the treatments involving malt diastase or Polyzime "P" produced results differing in any way from the ones soaked in water. The Clarase treatment, however, proved very beneficial. In Figure 1, A and B, are two groups of three cells each. The former is from the water treatment, and the latter following 22 minutes' immersion in Clarase. These two were taken at the same initial magnification of 450×. The Clarase treatment was used for periods ranging from 10 minutes to 17 hours. Exposures for as little as 10 minutes were effective, but 15 to 20 minutes appeared to give slightly better results while longer periods showed no improvement. The overnight treatment was too long, causing definite injury to the cells. Wash-

[3]Manufactured by the Takamine Laboratory, Clifton, New Jersey.

ing in water for at least 10 minutes following Clarase treatment usually resulted in darker staining.

The ineffective results secured when the diastatic enzymes were used alone suggested the possibility that proteolytic enzymes in the Clarase might have produced its beneficial effect. Accordingly, 1% solutions of pancreatin (supplied by the makers of Clarase), pH 7.02, and of papain, pH 5.35, were used in treatments similar to those described for Clarase. These treatments produced results definitely inferior to the water controls. A papain treatment of

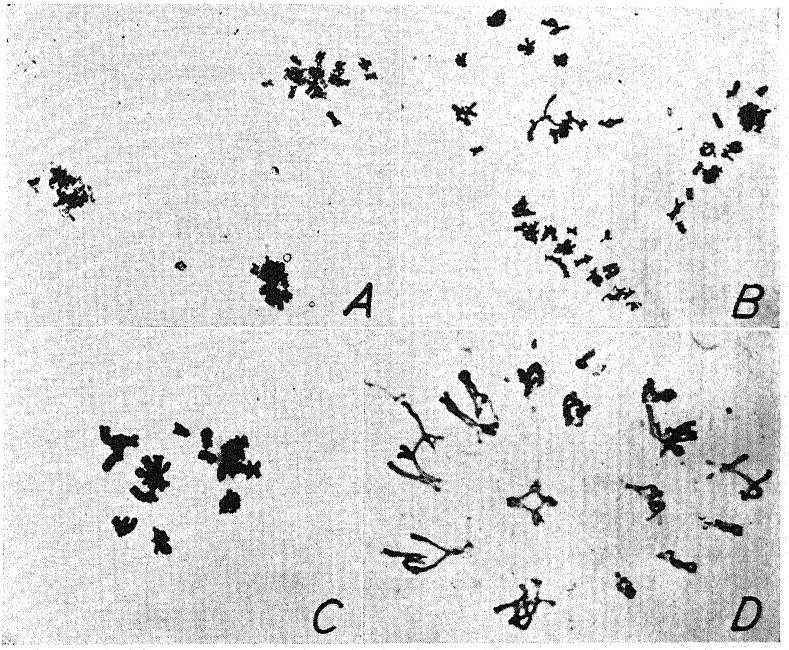

FIG. 1. First metaphase in tetraploid *Lilium longiflorum* following various treatments. (A) check from H_2O; (B) Clarase for 22 minutes; (C) papain for 27 minutes; (D) Clarase for 10 minutes. A, B 375×; C, D 510×.

27 minutes left the chromosomes clumped and the cells shrunken, as shown in Figure 1, C. (Compare it with Figure 1, D, a Clarase treatment of 10 minutes. Note the details of the chromosomes.) The pairing in this cell is easily determined as $9_{IV} + 5_{II} + 2_{I}$. Both cells are from the same anther and are at a magnification of 610×. Other series of treatments in which Clarase was used preceding or following malt diastase, Polyzime "P", pancreatin, or papain each proved less effective or no better than Clarase alone. Equal results were obtained with Clarase in water alone, pH 7.0, or when buffered at pH 5.0.

Frolova[4] has described the action of the enzymes of spleen juice (nucleases) on salivary gland chromosomes. Here the chromosomes were slightly reduced in size but there was no decrease in size of separate chromomeres. Later[5] pepsin treatments were used in conjunction with nucleases, and the spaces between the chromonemata were digested leaving the small chromomeres preserved. If the action was allowed to continue the entire chromosome structure was finally digested.

The recent studies by Greathouse, Klemme, and Barker[6] on the deterioration of cellulose by fungi, suggested that some of these organisms might prove useful in the present problem. Through the courtesy of G. A. Greathouse of this Bureau we were supplied with cultures of *Aspergillus niger* Van Tieghem, *Chaetomium globosum* Kunze, and a species of *Metarrhizium*. From 10 to 15 days after inoculation, the several flask cultures were extracted by grinding the contents of the flask with quartz sand in a mortar containing 10 ml. of a sodium acetate buffer, pH 5.0. The supernatant liquid was then used in a series of treatments on anther sections similar to those described for enzymes. Unfortunately our tetraploid material was all exhausted and diploid forms only were available.

All three fungus extracts produced beneficial results when the exposure period was not too extended. Four groups of cells from the same anther variously treated (A, water for 30 minutes; B, *Metarrhizium* for 18 minutes; C, *Chaetomium globosum* for 30 minutes; and D, *Aspergillus niger* for 33 minutes) are shown in Figure 2. All magnifications are 270×. In Figure 2, E, is shown the effect of 17 hours (overnight) in *Metarrhizium* extract. This is typical of results for the same exposure to the other two fungus extracts. In Figure 2, F, are shown cells from the same anther and treatment as E except that the anther section was washed in water for 1 hour and 45 minutes after removal from the *Metarrhizium* extract. Possibly the long exposure resulted in an accumulation of end products that was definitely harmful but readily removed by washing.

In a third series of tests Clarase and the fungus extracts were used on anthers from the triploid *L. tigrinum*. Each anther was cut into two equal parts, one being placed in normal extract and the other in the same extract after it had been boiled. Typical results are shown

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[4]Frolova, S. L. C. R. Acad. Sci. URSS, XXX, No. 5, 1941.

[5]————. Fine chromosome structure after enzyme action (nuclease and pepsin). Comptes Rendus de L'Acad. Sci. de L'URSS, **32**, 654–7. 1941.

[6]Greathouse, G. A., Klemme, D. E., and Barker, H. D. 1942. Determining the deterioration of cellulose caused by fungi. Ind. and Eng. Chem., Anal. Ed., **14**, 614–20.

in Figure 2, G, H, I. The magnification is 270×. In Figure 2, G, the cells are from the water treatment; in H from Clarase; and in I from the boiled Clarase. Similar results were secured from the tests with the fungus extracts, indicating that heat inactivated the agents responsible for the beneficial effects of the fungus extract.

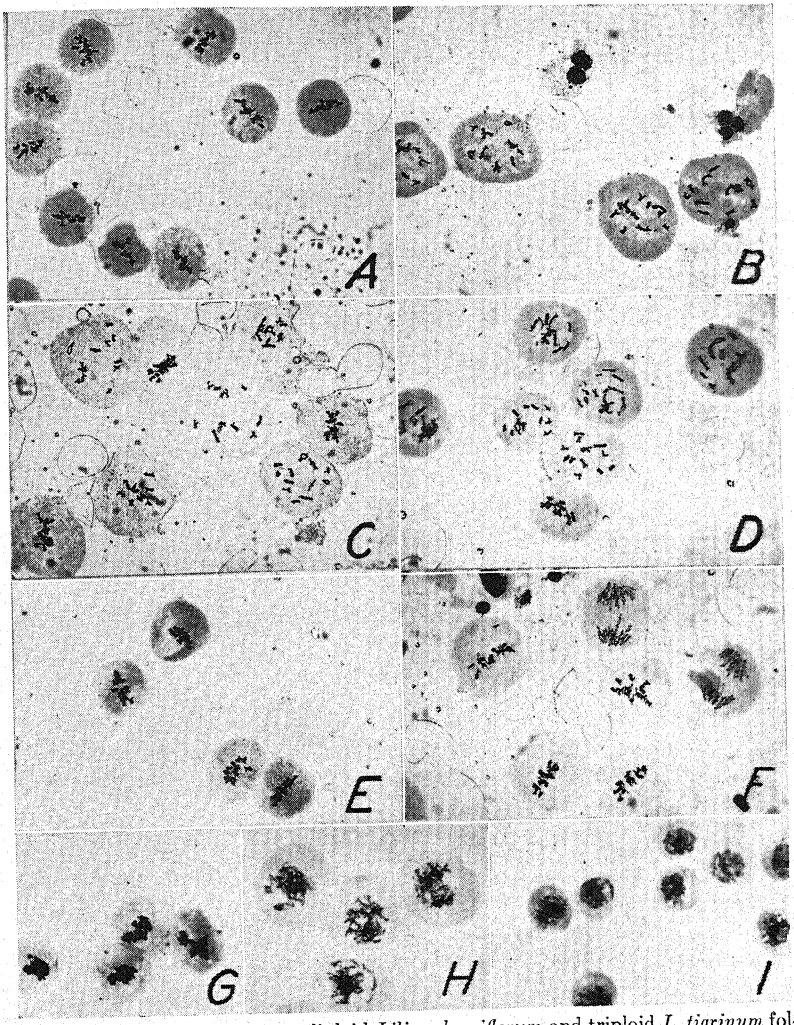

FIG. 2. First metaphase in diploid *Lilium longiflorum* and triploid *L. tigrinum* following various treatments. *L. longiflorum:* (A) control from H_2O after 30 minutes; (B) *Metarrhizium* sp. extract for 18 minutes; (C) *Chaetomium globosum* extract for 30 minutes; (D) *Aspergillus niger* extract for 33 minutes; (E) *Chaetomium globosum* extract for 17 hours; (F) same as (E) except washed in H_2O for 1 hour and 45 minutes before staining. *L. tigrinum:* (G) control from H_2O after 30 minutes; (H) Clarase 20 minutes; (I) boiled Clarase 20 minutes. All 235×.

Some observations have also been made on the effectiveness of these treatments in preparation of root tip smears. The three fungus extracts had some effect on the middle lamella, making possible the separation of the cells. The 1% Clarase solution did not have any appreciable effect, so a 5% solution was tried. The root tips were treated in the Clarase for various intervals from 10 to 45 minutes. They were then washed in water for about 10 minutes prior to staining. So far the results with root tip smears have not been promising.

The results presented in this paper are concerned only with species of *Lilium*. Clarase, however, has also now been used by A. E. Clarke, of this Division, on the pollen mother cells of an amphidiploid *Allium*. Prior to its use great difficulty was encountered in analyzing chromosome pairing. While the methods for using enzymes are not yet standardized, repeated preparations have produced the same effects. It is very necessary, of course, that all fixed material be thoroughly washed in water before treating with any of the materials mentioned in this paper. The results may vary even on the same slide, but this is to be expected since different phases of the meiotic cycle may be affected in various ways. The enzyme responsible for the beneficial effects shown by the Clarase and fungus extracts has not yet been identified. Furthermore, little is known concerning optimum concentration, pH, and stability of the extracts.

BODIAN'S PROTARGOL METHOD APPLIED TO OTHER THAN NEUROLOGICAL PREPARATIONS

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ABSTRACT.—Some applications of the Bodian technic for other than neurological purposes are described. After fixation for 48 hours, in formalin, 5 ml., acetic acid, glacial, 5 ml., and 80% ethyl alcohol, 90 ml., the routine procedures are recommended, with the exception that the exposure to protargol for 24 hours and subsequent reduction should be repeated once. Gold toning may be omitted. With this method the argentaffin (chromaffin) cells of the digestive tract (rat, hamster), the alpha cells of the pancreatic islands (hamster) and the medullary cells of the suprarenal gland (hamster) are selectively impregnated. In the mammalian pituitary gland (rat, hamster) certain of the granulated chromophile cells are impregnated. In the rat only the basophiles appear to react with the silver.

The purpose of this brief note is to direct the attention of histologists to the value of Bodian's (1936, 1937) protargol method for the demonstration of certain specialized cells of vertebrate organs. Its value as a general histological method, frequently equal to routine hematoxylin and eosin preparations, has long been recognized, but in addition it frequently impregnates selectively certain glandular elements and furnishes some striking staining contrasts. However, in most instances there are other adequate methods for the differential staining of such elements, and the chief advantage is in the contrast provided. Other advantages are that it is a silver method which may be applied to mounted paraffin sections and that its widespread use in American laboratories for neurological studies makes it a familiar and readily available method for these secondary purposes.

The technical procedure outlined by Davenport, Windle and Rhines (1943) was followed carefully using the fixative recommended (formalin, 5 ml.; acetic acid, glacial, 5 ml.; 80% ethyl alcohol, 90 ml.) for 48 hours. The exposure to protargol (24 hours) and subsequent reduction were repeated once, as a single treatment did not adequately blacken the intracellular granules. The explanation of the advantage of the double treatment is not known but it may be related to the slowly changing pH of the protargol solution

after metallic copper has been added. This problem of optimum pH for nerve axon impregnation has been recently discussed by Holmes (1943).

Toning with gold chloride may be omitted without seriously impairing the preparations as far as the demonstration of the impregnated elements is concerned, but the nuclear and cytoplasmic detail of the rest of the tissue is not so clear. Without toning, the cytoplasmic background is golden yellow with dark brown nuclei. After toning the color varies with the type of tissue and the duration of the toning process. The shades of cytoplasm run from lavender to purple or reddish brown. The nuclei usually appear black.

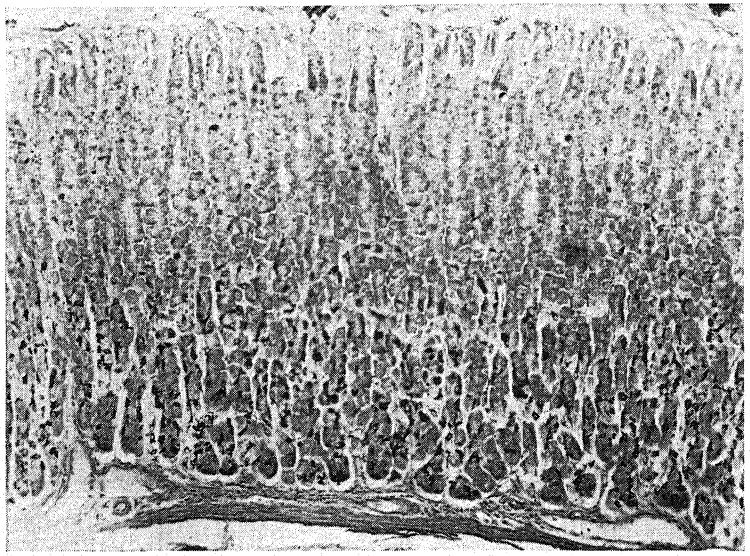

FIG. 1. Vertical section through the fundic mucosa of the rat, showing the number and distribution of the enterochromaffin (argentaffin) cells. They are confined chiefly to the inner one-half of the mucosa but scattered cells occur all the way to the gastric foveolae (Bodian impregnation, 15μ thick, $\times$96).

Bodian's method was first used on the gastric mucosa of the rat in an effort to find some uniform method of impregnating the intracellular canaliculi of the parietal cells rather than depend on the uncertain results of the rapid Golgi method. However, the intracellular canaliculi were not demonstrated by this method but the enterochromaffin cells of the mucosa were beautifully impregnated (Fig. 1). Subsequently this method was also used on preparations of the small intestine of the rat as well as the stomach and intestine of the hamster. In all instances the enterochromaffin cells were

typically shown as by the older, specific methods of Masson and Hamperl. See Lison (1936) for a discussion of these methods and Macklin and Macklin (1932) for a general discussion of the enterochromaffin cells.

The method was also applied to the pancreas, suprarenal gland and the anterior pituitary of the hamster. In the pancreas the impregnated cells were limited to the periphery of the pancreatic islands and careful comparison with selectively stained preparations (Heidenhain's azan method) indicated that the silver impregnation was limited to the alpha cells only, leaving the beta cells unblackened and stained similarly to the exocrine tissue. A somewhat comparable silver impregnation of the aplha cells of the avian pancreas has been reported by Nagelschmidt (1939) but in this instance these cells could not be impregnated by the Masson-Hamperl method although they reacted readily to another silver method, the Gros-Schultze technic.

In the suprarenal gland only intracellular granules of the chromaffin cells of the medulla are blackened by the silver. The cytoplasm of the sympathetic nerve cells and the cortical cells is a deep lavender with dark nuclei. Peculiarly enough, the nuclei of the medullary cells are completely unstained and appear as clear spaces in the midst of the densely granulated cytoplasm. Bennett (1941) also reports successful staining of the suprarenal medulla of the cat by Bodian's method following Bouin's fixation, but in his illustrations the nuclear patterns are clearly shown.

Some preliminary observations on the anterior lobe of the pituitary of the hamster and the rat indicate that this silver impregnation method may have a limited use in demonstrating certain stages of activity of the chromophile cells. In the hamster the picture of impregnation appears rather variable and it has not been possible to determine the relationship of the argentaffin cells to the cyclic changes of either the acidophiles or the basophiles. In the rat, however, the silver reaction appears to be more specific for the basophiles, at least in the heavily granulated stages. The possible relationships of the argentaffin cells demonstrated by another silver method to the cellular population have been recently discussed by Popoff (1943) for the pituitaries of rabbit and man.

A more complete survey of the potential uses of the Bodian technic on other tissues or organs might considerably extend the applications of this method. The diversity of cells impregnated suggests that this silver reaction is a rather general one, probably not dependent on a specific chemical constituent or physical condition within the individual type of cell.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

DYES AND THEIR BIOLOGICAL USES

BURCKHALTER, J. H., JONES, E. M., HOLCOMB, W. F., and SWEET, L. A. **N-substituted 2-methoxy-6-chloro-9-aminoacridines.** *J. Amer. Chem. Soc.*, **65**, 2012. 1943.

The preparation and properties of 25 N-substituted 2-methoxy-6-chloro-9-aminoacridines have been described. These compounds are dialkylaminoalkylaminoacridines, aromatic and heterocyclic aminoalkylaminoacridines and miscellaneous N-substituted aminoacridines. Nearly all of these compounds have been tested for their antimalarial action against *Plasmodium cathemerium* in canaries by Professor A. L. Tatum of the University of Wisconsin, who will report the results elsewhere.—*A. P. Bradshaw.*

GALAT, ALEXANDER. **New processes for sulfanilamide.** *Ind. and Eng. Chem., Ind. Ed.*, **36**, 192. 1944.

Two new processes have been described for the manufacture of sulfanilamide which are simpler and shorter, and which involve raw materials less vital to the war effort than those previously used. Formic acid is substituted for acetic acid in the first process, and urea in the second.—*A. P. Bradshaw.*

KUMLER, W. D., and DANIELS, T. C. **The relation between chemical structure and bacteriostatic activity of sulfanilamide type compounds.** *J. Amer. Chem. Soc.*, **65**, 2190. 1943.

Evidence is presented, first, that a fundamental factor for activity of sulfanilamide type compounds is the contribution of the resonating form with a coplanar amino group; second, that the negative character of the SO_2 group is a concomitant factor associated with the resonating form; third, that compounds which appear to be exceptions to Bell and Robbin's theory, or do not fall within the scope of their theory, can be adequately accounted for on the basis of resonance; and fourth, that these ideas, in part, apply to certain other bacteriostatic compounds including the monoamino acridines.—*A. P. Bradshaw.*

KWARTLER, C. E., and LUCAS, PHILIP. **The preparation of sulfanilamidoindazoles.** *J. Amer. Chem. Soc.*, **65**, 1804. 1943.

The high chemotherapeutic activity of the sulfanilamido derivatives of various aminoheterocycles (e.g., sulfathiazol, sulfadiazine, sulfamethazine and sulfapyridine) suggested the preparation of various isomers of sulfanilamidoindazole in order to obtain information on the effect of substitution of the sulfanilamido group in different positions in the indazole ring. The 3-, 5-, 6- and 7-sulfanilamidoindazoles and the necessary intermediates for these compounds were prepared. These compounds were found to have distinct bacteriostatic and bactericidal properties against a variety of organisms. The 5-sulfanilamidoindazole and 6-sulfanilamidoindazole are twice and 3 to 4 times, respectively, as active as sulfanilamide against streptococcus. The toxicity of these compounds appears to be low. Other pharmacological data are being collected. The chemotherapeutic studies will be reported elsewhere in detail.—*A. P. Bradshaw.*

MUELLER, A. C., and HAMILTON, C. S. **The synthesis of 1-substituted aminobenzo(f)quinolines.** *J. Amer. Chem. Soc.*, **65**, 1017. 1943.

In connection with the work on synthetic antimalarials, several 1-substituted aminobenzol(f)quinolines and the necessary intermediates for these compounds have been prepared.—*A. P. Bradshaw.*

POPKIN, A. H. **Derivatives of biphenylsulfonamides. I. Preparation of p-(o-aminophenyl)-benzenesulfonamide.** *J. Amer. Chem. Soc.*, **65**, 2043. 1943.

The present study was undertaken to determine the chemotherapeutic value of some sulfonamide derivatives of biphenyl. The first paper of the series describes only the preparation of the parent substance, p-(o-aminophenyl)-benzenesulfonamide, and establishes the position of the sulfonamide group.—*A. P. Bradshaw.*

POPKIN, A. H., and PERRETTA, GERTRUDE M. **Derivatives of biphenylsulfonamide. II. Derivatives of p-(o-aminophenyl)-benzenesulfonamide.** *J. Amer. Chem. Soc.*, **65**, 2046. 1943.

Six derivatives in which a hydrogen on the sulfonamide nitrogen of p-(o-aminophenyl)-benzenesulfonamide is replaced by an aromatic group have been made. Bactericidal tests on the parent substance and these derivatives indicate that these compounds are inactive *in vitro* against *Escherichia coli* and *in vivo* against streptococcal infected mice. Since it was previously reported by Kumler and Halverstadt that p-(p-aminophenyl)-benzene sulfonamide was inactive, the conclusion was drawn from the present work that the shift in the position of the amino group does not increase activity.—*A. P. Bradshaw.*

SHREVE, R. N., and BENNETT, R. B. **Studies in azo dyes. I. Preparation and bacteriostatic properties of azo derivatives of 2,6-diaminopyridine.** *J. Amer. Chem. Soc.*, **65**, 2241. 1943.

The hydrochloride of 2,6-diamino-3-phenylazo-pyridine, known in the trade as "Mallophene" or "Pyridium", is being used extensively at the present time as a urinary antiseptic. A series of 30 dyes, derivatives of 2,6-diaminopyridine, was prepared, and their bacteriostatic and other properties were tested, in order to determine any correlation between these data and their chemical structure. The organisms used in the bacteriological tests were *Escherichia coli* and *Staphylococcus aureus*. Using phenylazo-diaminopyridine as a reference dye, several of the dyes prepared were found to have greatly enhanced bacteriostatic indices, others were lower. Methylation, either alone or in combination with nitro groups, appears to increase bacteriostatic activity. In general, methoxy groups are less effective. When the dye molecule becomes too large, the bacteriostatic activity becomes suppressed. Increasing the complexity of the molecule reduces its solubility in water.—*A. P. Bradshaw.*

SHREVE, R. N., and BENNETT, R. B. **Studies in azo dyes. II. Preparation and bacteriostatic properties of azo derivatives of 8-quinolinol.** *J. Amer. Chem. Soc.*, **65**, 2243. 1943.

A series of 28 azo derivatives of 8-quinolinol were prepared. Bacteriostatic tests against *Escherichia coli* and *Staphylococcus aureus* show these compounds to be highly bacteriostatic. No definite indications of correlation between chemical structure and bacteriostatic activity have been found. The low solubility in water of some of the dyes having rather strong activity limits their use in certain directions, but such insolubility may be desirable under some circumstances to prevent too rapid physiological elimination. These dyes appear to have no value as textile dyes.—*A. P. Bradshaw.*

SIEBENMANN, C., and SCHNITZER, R. J. **Chemotherapeutic study of p-nitrobenzoyl- and related compounds.** *J. Amer. Chem. Soc.*, **65**, 2127. 1943.

The p-nitrobenzyl and 3,5-dinitrobenzoyl derivatives of a number of aromatic and hydroaromatic hydroxy and amino compounds were prepared and tested for chemotherapeutic activity in mice infected with hemolytic streptococci, pneumococci and meningococci. The 3,5-dinitrobenzoyl derivatives showed no activity; the p-nitrobenzyl esters of 4-hexylresorcinol showed some activity against pneumococci. In a series of sulfanilamide derivatives prepared, the N′-p-nitrobenzoyl compound showed a lower meningococcal activity but was active in pneumococcal infections, although less so than sulfadiazine. The di-substituted N′,N⁴-compounds were completely inactive.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

BARRETT, A. M. **A method for staining sections of bone marrow.** *J. Path. & Bact.*, **56**, 133–5. 1944.

The following method allows satisfactory staining of bone marrow with less dependence on condition of tissue, method of fixation, and decalcification, and is also suitable as a general stain:

Fix in formol-saline 12–24 hr., followed by Zenker-formol 24–48 hr. Either fixative may be used alone. Decalcify either by the method of R. P. Custis (Amer. J. Med. Sci., **185**, 617) or by the magnesium citrate method (Shipley, P. G., in McClung's Handbook; *cf.* p. 60 in Cowdry, E. V.: Microscopic Technique in Biology and Medicine). Cut paraffin sections as thin as possible. Stain in Carazzi's hematoxylin 10–20 min. (water, 400 ml.; glycerol, 100 ml.; potassium alum, 25 g.; potassium iodate, 0.1 g.; hematoxylin crystals, 0.5 g. Mix and dissolve without heat). Rinse in tap water and differentiate in acid alcohol (alcohol, 70 ml.; concentrated HCl, 1 ml.; water, 29 ml.) 10 sec. Wash in running tap water 5 min. or more.

To assure improved staining of fine acidiphil granules, as in human neutrophiles, the author recommends a counterstaining procedure calling for the following solutions: Orange mixture: 1% aqueous erythrosin, 1 vol.; 1% aqueous orange G, 3 vol.; water, 2 vol. Buffer solution, consisting of a mixture of: (A) 4.0 ml. *N* KH_2PO_4 in 250 ml. distilled water, and (B) 1 ml. *N* NaOH and 1 ml. *N* KH_2PO_4 in 250 ml. distilled water; A and B should be mixed in ratios varying from 2:1 to 1:2; the larger the amount of A used the more the red is accentuated, while B accentuates the blue. The procedure of staining is as follows: Support the slide on matches in a petri dish and pour on a mixture of 0.5 ml. of the above orange mixture, 0.5 ml. of the buffer solution and 1.0 ml. of acetone. (This quantity is suitable for a 3 $\times 1''$ slide.) Cover the dish and stain for 20–30 min. Rinse in running tap water, then distilled water.

Whether or not the previous staining procedure has been performed, one should continue as follows: Transfer sections to a staining jar containing a freshly prepared staining mixture consisting of water, 30 ml.; buffer, 10 ml.; orange mixture, 0.1 ml.; blue mixture (1% aqueous methylene blue, 2 vol.; 1% aqueous toluidine blue, 1 vol.; water, 17 vol.). Use the same buffer found suitable in the previous staining step. Stain 18–24 hr. (The stain should not change color, or at most only slightly during staining.) Dehydrate in absolute alcohol and clear in xylene. Blot off most of the xylene and mount in Gurr's neutral mountant.

If sections are too blue, they may be improved by removing the cover slip and rinsing with alcohol, which dissolves some of the mountant and removes some of the blue. Erythrocytes stain pink or orange-pink, the cytoplasm of other cells deep blue to colorless, nuclei gray-blue, the granules of neutrophils crimson, eosinophil granules orange to scarlet, and the granules of basophils purple-blue.

To prevent mold growth the buffer should be stored in the refrigerator.—*S. H. Hutner.*

BARRETT, A. M. **On the removal of formaldehyde-produced precipitate from sections.** *J. Path. & Bact.*, **56**, 135–6. 1944.

Formaldehyde acting on blood produces a brownish-black precipitate which is hard to remove without also removing sections from the slide. This precipitate may be eliminated without washing sections off the slide by placing them, after removal of paraffin, into a saturated solution of picric acid in alcohol. Two hours' treatment is ample; frequently half this time suffices. The picric acid is then removed by a brief rinsing in tap water or mildly alkalinized water, and one can proceed with staining etc. as usual.—*S. H. Hutner.*

CHANG, MIN-CHUEH. **Disintegration of epidymal spermatozoa by application of ice to the scrotal testis.** *J. Exp. Biol.*, **20**, 16–22. 1943.

In order to stain spermatozoa for counting, semen was diluted with the following solution, freshly prepared before use: 180 ml. NaCl, 0.9%, dissolved in 10 ml. KOH, 2% for rabbit, or in 20 ml. for rat and guinea pig; Loeffler's methylene blue, 3 ml. Since Loeffler's methylene blue contains KOH, this solution simply increases the concentration of KOH to an optimum for the present purpose.—*C. E. Allen.*

ERCOLI, N., and LEWIS, M. N. **The age factor in response of bone tissue to alizarin dyes and the mechanism of dye fixation.** *Anat. Rec.*, 87, 67–76. 1943.

The mechanism of dye fixation in bone tissue has been studied for over two hundred years. First madder, later alizarin and still later sodium alizarin sulfonate were used for coloring. It was believed from the early studies that the dye was fixed in the deposited calcium salts.

Subcutaneous and intravenous injections of varying amounts of alizarin red S dye in growing and adult albino mice were made. To get 50% coloration in adult mice bone, two to three times greater doses were required than in growing mice. Intravenous injections in the adult mice gave better coloration.

The mechanism of fixation in the bone tissue, from the above experimental results, is adsorption between the calcium phosphate in the mouse bone tissue and the dye anions and not the formation of calcium alizarinate or a reaction with free calcium ions.

Lesser doses of the alizarin red S dye are required for younger mice bone because there is a higher vascularization and a higher permeability.—*W. R. Hunt.*

HESS, MANFRED, and HOLLANDER, FRANKLIN. **Permanent metachromatic staining of gastric mucus smears.** *J. Lab. and Clin. Med.*, 29, 321–3. 1944.

The following procedure produces metachromatic stains which are permanent for at least 10 months: Spread the mucus gently on a slide with a glass rod, air-dry at room temperature and fix by flaming. Place in toluidine blue solution (dissolve 1.0 g. of toluidine blue, certified, in 90 ml. of distilled water and filter; add 0.5 ml. of HCl specific gravity 1.18, 0.9 ml. of H_2SO_4 specific gravity 1.84, 10 ml. of 1% alum, and 5.0 ml. of 95% alcohol). Agitate for 15 sec. and let stand 1 min. more. Pass through 2 changes of HCl (1 ml. HCl specific gravity 1.18 and 9 ml. of water) or until no more color runs. Immerse for 1 min. in 5% $HgCl_2$, wash vigorously (about 5 seconds) in 2 changes of 95% alcohol and 2 of xylene. Mount in balsam. Both extra- and intra-cellular mucus will be stained red to purplish red and the cells blue, nuclei being a deeper blue.—*John T. Myers.*

MILLER, JOHN A. **A new method of staining nervous tissue.** *Ohio J. of Sci.*, 44, 31–5. 1944.

This technic employs elements of the Golgi method and Cajal's reduced silver method to differentiate the histological and cytological details of the leech's nervous system.

Sections of a completely dissected nerve trunk of a leech, which has been fixed in 10% formalin, dehydrated in graded alcohols, and embedded in celloidin, are stained in either of the two following ways:

Variant A, for best histological study: Gradually hydrate and place in 6% $AgNO_3$ for 4 days in the dark. Wash well in distilled water. Reduce for 24 hr. in a mixture of hydroquinone, 2 g.; neutral formalin, 20 ml.; distilled water, 200 ml. Wash well in distilled water. Tone 30 min. in 0.2% gold chloride. Wash in distilled water. Fix 30 sec. in 5% $Na_2S_2O_3+5H_2O$ and wash in distilled water. Counterstain at this point, if desired. Dehydrate, clear, and mount in clarite.

Variant B, for best cytological study: Gradually hydrate and place in 20% $AgNO_3$ for 60–90 min. Wash quickly in distilled water. Place for 3 min. in freshly prepared ammoniated $AgNO_3$ made as follows: add concentrated NH_4OH drop by drop to 20% $AgNO_3$ solution until the precipitate is almost dissolved; filter. Wash quickly in distilled water. Place 1 min. in 10% formalin and wash well in distilled water. Tone 10 min. in 2.0% gold chloride and wash with distilled water. Reduce 20 sec. in the following mixture: oxalic acid, 2.0%; formalin, 1 ml. per 100 ml. of solution. Wash in distilled water. Fix 2 min. in 5.0% $Na_2S_2O_3+5H_2O$ and wash in distilled water. Counterstain, if desired. Dehydrate, clear, and mount in clarite.—*C. Randall.*

STAIN TECHNOLOGY

VOLUME 19 NOVEMBER, 1944 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

RECENT ACTIVITIES OF THE STAIN COMMISSION

History of the Commission. The early history of the Stain Commission was discussed in the first issue of STAIN TECHNOLOGY,* where it is available to anyone interested. The only reason for summarizing it now is that the Commission was recently reorganized, and some understanding of its history is necessary to put its present status into the proper background.

When the first World War ended it was found that the German stains upon which biologists had previously been dependent were no longer available in America; and laboratory workers who had been forced to use products of American manufacture in Army camps and hospitals during the war reported that they had not been able to obtain the same results as they were used to in the past. To put it mildly, their remarks about American stains were not exactly complimentary. Most of these complaints came from bacteriologists or pathologists, and many of them reached the ear of Col. A. P. Hitchens, who was at that time Secretary-Treasurer of the Society of American Bacteriologists. He referred the matter for investigation to that Society's Committee on Bacteriological Technic, of which the writer of this note happened to be Chairman.

The original idea of the investigation was to confine it to bacteriological stains and to have it conducted by members of the Society. It was soon realized that other fields of biology were equally involved, and the problem was taken up by the National Research Council. The latter organization, in November, 1921, appointed a Committee on Standardization of Biological Stains, which functioned a year and then turned over its work to a newly formed organization known as the Commission on Standardization of Biological Stains. The Commission, as originally constituted, consisted primarily of the collaborators who had been appointed by the original committee to assist in its investigations; the Executive Committee of the Commission, on the other hand, was composed of representatives appointed by several national scientific organizations covering fields in which biological stains are em-

ployed. Originally there were five such society representatives, but their number was later increased to eight, to include other fields of science. Essentially this form of organization remained unchanged until early in 1944.

Finances. The original investigations, as planned under the Research Council, were modest in scope, and called for no high finance to organize them. For the small amount needed to hire necessary clerical work, the coöperation of the Chemical Foundation was secured. Its president, the late Mr. F. P. Garvan, proved very much interested in the work, and as the activities of the Commission expanded, his organization contributed larger and larger annual sums, so that the Commission was able to hire its own employees to do much of the testing, instead of having to depend wholly on voluntary collaboration. In all, the Foundation must have contributed about $100,000 to this work.

Meanwhile the Commission had slowly been building up an income of its own, partly from the sale of publications but largely from fees charged to manufacturers of biological stains. During the period from 1931 to 1938 the earned income was increasing and the contributions from the Chemical Foundation decreasing. Early in 1939 the latter stopped entirely, because of the expiration of patents on which the Foundation had been dependent. Fortunately this year was also the one in which the earned income of the Stain Commission became practically enough to balance its budget.

Reorganization. Largely because of this changed financial status, it seemed that the organization should be incorporated. Steps looking toward this incorporation were taken during 1942 and 1943. The actual incorporation took effect on Feb. 21, 1944, as explained in the footnote to the recently published bylaws of the corporation. This incorporation took effect under the terms of the charter granted by the University of the State of New York.

According to this charter the organization now becomes known as the Biological Stain Commission, Inc., which is organized as a non-profit corporation to take over the duties and assets of the Commission on Standardization of Biological Stains. The members of the Executive Committee of the defunct organization become trustees of the corporation with the addition of three new members, bringing the total number of members of the Board of Trustees up to eleven. Between meetings of the Board, the affairs of the Commission are managed by an Executive Committee of three. The officers consist of President, Vice-President, Secretary and Treasurer.

Membership qualifications. At the time of meeting for reorganiza-

tion, two important committees were appointed. One of them, of which Dr. R. D. Lillie is chairman, is a Membership Committee. The object of this committee is to establish qualifications for membership. As already explained, the original membership of the Commission consisted of those collaborators who had taken part in the early investigations. There were about 60 of them. As this original membership decreased, invitations were extended informally to others who were known to be interested in the work, and in that way the membership was maintained at approximately its original figure. It was felt, however, that the membership of the new organization should be put on a more satisfactory basis. It was agreed that its charter members should be those of the old Commission who were still active; it was also agreed that others should be invited, and the Membership Committee was appointed to determine qualifications and to pass upon new members that would be proposed. After a meeting, this committee recommended that members should be admitted only on invitation, that each new member should be proposed by one of the trustees, seconded by another trustee, and then a statement of his qualifications, accompanied by a list of publications in regard to dyes, staining, or microtechnic in general, should be submitted to the Membership Committee for action. This recommendation was adopted.

Plans for Research. The other committee appointed may well prove more important. It was a Research Committee, under the chairmanship of Dr. W. F. Windle. The object of this committee is to determine desirable fields for research along the lines of interest to the Commission and to decide how to organize work in those fields. This committee has been carrying on considerable correspondence since the meeting and is working on a report to submit to the Board of Trustees. It is hoped that future issues of STAIN TECHNOLOGY may have further statements as to the activities to be stimulated by this committee.

Publications. The demand for the book BIOLOGICAL STAINS has proved so great, since the United States entered the war, that the fourth edition is now out of print. Work on the fifth edition is in progress; but as more changes are contemplated than have been made in any recent edition, it is taking considerable time to get the revised manuscript in final shape. This fact, together with the delays in printing that are to be expected under present conditions, makes the date of publication of the new edition quite uncertain.

Meanwhile the Commission is sponsoring the publication of a

new manual, known as Staining Procedures (by H. J. Conn and Mary A. Darrow). This is a loose-leaf publication, designed to contain methods that are employed by members of the Commission, which is being issued, leaflet by leaflet, as they are completed. At the present time seven leaflets have been published, and two more (which will probably complete the series) are almost ready. (See advertising pages for more information.) After the entire series has been completed it is planned to keep the publication up to date by occasionally revising leaflets or issuing supplementary pages to include additional methods. In this way it is hoped to keep Staining Procedures a living publication that is not continually getting behind the times.

The Future. It is hoped that the new form of organization will allow greater developments in the future than the past has seen. Just what they will be, it is still hard to say; but there is no question but that there is a field for research and for standardization activities in regard to other stains and related products that have not yet been covered by the work of the Commission. Suggestions from anyone interested will be welcome.—H. J. CONN.

PREPARING SPECIMENS OF BONE AND TEETH FOR CUTTING BY THE PARAFFIN METHOD

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ABSTRACT.—A method of preparing bone or teeth for sectioning is described which involves the following steps: 48 hr. in 1:10 formalin; 24 hr. in 70% alcohol; decalcification for several days in 10% HNO_3; rinsing and transferring to 2% potassium alum for 12 hr.; rinsing and treating with 5% $NaHCO_3$ (or Li_2CO_3) for 24 hr.; washing for 12–24 hr.; then passing through ascending grades of alcohol to xylene. In the case of developing teeth, a slightly different procedure is recommended: fixation in Heidenhain's SUSA till hard tissue is decalcified; 24 hr. in 96% alcohol (with three changes); 24 hr. in absolute alcohol (with one change); clearing in xylene or chloroform, and embedding in paraffin.

The demonstration of the minute structure of bone and teeth, just as is the case with other tissues of the body, requires quite thin sections, which will still possess good staining qualities, despite the fact that the tissues have been decalcified.

Because of the many difficulties associated with the making of paraffin sections of bone and teeth, until recently the cutting of these tissues has customarily been carried out by use of the freezing or celloidin methods.

MATURE BONES AND TEETH

The present communication gives an account of a method by which specimens of bone and teeth can be brought through to paraffin, thus allowing the cutting of thin sections with a fair degree of certainty.

It is not claimed that the method is new in its entirety, but it *is* claimed that if the processes be carried through as described, reliable results should be obtained.

Most of the specimens prepared were stained by the Azan or Picro-Mallory (Lendrum and McFarlane, 1940) technics, with modifications where necessary. Excellent pictures of adult and developing teeth have been obtained, the detail and contrast being far superior to that given by the ordinary hematoxylin and eosin methods. In addition, it has been found that even the somewhat capricious Schmorl's method gives more certain results with a

greater degree of brilliance in staining than are obtained with the freezing and celloidin methods. One of the most important features of the method is that there is little or no shrinkage of the soft tissues away from the hard tissues; for example, the soft and delicate tissue of the pulp of the tooth, with its lining border of odontoblasts lies in perfect apposition to the dentine, giving an undistorted picture of their relationship.

Method: The specimens of bone and teeth are first of all sawn to suitable dimensions before placing them in the fixative. Specimens which will show bone and teeth at the same time are obtained by sawing through the lower jaw of a cat or kitten in the spaces between the teeth and placing the pieces in the fixative. They are then treated as follows:

(1) Fix for at least 48 hours in 1:10 formalin.
(2) 70% alcohol 24 hours.
(3) Transfer to 10% HNO_3. Leave till thoroughly decalcified, changing the acid every second day. The time of decalcification will of course depend on the size of the piece of tissue; always trim away as much excess tissue as possible.
(4) Rinse in one or two changes of water, a few seconds only, and transfer to 2% potassium alum; leave for 12 hours.
(5) Again rinse in water, transfer to 5% $NaHCO_3$ or Li_2CO_3 (half saturated) for 24 hours.
(6) Wash in running water using a washing bobber (Gairns, 1942) for 12–24 hours. The tissues are now ready for carrying through to paraffin. The time-table given here will be found to be most convenient for the routine day of the laboratory
(7) 10% alcohol—9 a.m. to 12 noon.
(8) 20% alcohol—12 noon to 5 p.m.
(9) 40% alcohol—overnight.
(10) 60% alcohol—9 a.m. to 12 noon.
(11) 80% alcohol—12 noon to 5 p.m.
N.B. It is an advantage to add to these alcohols phenol up to 6% (Lendrum, 1935).
(12) Absolute alcohol, overnight until 10.30 a.m., changing the alcohol at 9 a.m.
(13) Xylene, 10.30 a.m. to 12 noon.
(14) 52°C. paraffin, 12 noon to 2.30 p.m.; then 60°C. paraffin until 4.30 p.m., changing once.
(15) Embed and cool.

Any of the usual clearing agents may be used instead of xylene. Benzene, butyl alcohol, cedarwood oil, and aniline oil, have been

tried without any noticeable difference. Xylene seems to require a shorter time for clearing which is perhaps of some slight advantage. After the paraffin block has been trimmed and fixed to the nosepiece of the microtome, it should be allowed to stand in very cold water for about 10 minutes. This makes an astonishing difference to the cutting of the sections.

Developing Teeth

In the case of developing teeth, a different procedure is required. Undoubtedly the finest fixative for jaws of young animals is Heidenhain's Susa, formula:

Saturated aqueous $HgCl_2$	50 ml.
Formalin	20 ml.
Glacial acetic acid	4 ml.
Distilled water	30 ml.
Trichloracetic acid	2 g.

It should be noted that Susa fixes and decalcifies at the same time. It is perhaps a little slow in its decalcifying action but the time involved is well worth while as the end results give eminent satisfaction. It is also one of the best fixatives to use if aniline dyes are to be employed for staining.

Method:

(1) If embryo jaws are used, immerse in Susa till the hard tissue is decalcified. If the specimens are taken from animals after birth, extra trichloracetic acid (up to about 10%) can be added to the Susa solution. This accelerates the decalcification and at the same time is not too drastic.
(2) Transfer direct to 96% alcohol; change three times in 48 hours.
(3) Absolute alcohol 24 hours; change once.
(4) Xylene or chloroform until clear.
(5) Embed in paraffin, 4 hours at 52°C.

Note:—Since Susa contains $HgCl_2$, the sections must of course be treated with iodine and thiosulfate before staining.

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LA TECHNIQUE DE TANNIN-FER

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ABSTRACT.—The author points out that he should be accredited for originating the tannic-acid-iron method for staining tissues. Reference to *Comp. Rend. Soc. Biol.*, volume 83, 1920, shows that Salazar introduced the method at that time. Several writers in recent years have failed to acknowledge this work.

The author has used this technic in studies of the Golgi apparatus in the ovary and the thyroid gland. In the field of hematology, he has combined the tannic-acid-iron method with Giemsa stain and claims that his method allows a sharper differentiation between agranulocytes and granulocytes, as well as a satisfactory revision of the Arneth classification of neutrophils.

More recently the author has devised several new modifications of the tannic-acid-iron technic which he has not yet published. He feels that these modifications should be a distinct aid to cytology and histology. He concludes: "One might say that the tannic acid and iron reaction has already created a new chapter in histological technic, a chapter which is not yet closed."

La méthode au tannin-fer a été introduite par nous dans la technique en 1920 (1), ce que plusieures auteurs, Rösler, Zimmermann, Korody, Härtel et encore récemment Sharman, semblent ignorer (2).

Nous avons montré dans une série de travaux publiés après 1920 les résultats qu'on peut obtenir en général avec cette réaction et particulièrement ceux qu'on obtient dans l'ovaire et dans l'analyse du complexus de Golgi, etc., tandis que mon élève Bacelar étudia les résultats de la même technique dans l'étude de la thyroide (3–18).

Nous avons essayé de débrouiller la question du mordançage tannique, et nous avons surtout mis en relief que la réaction tanno-férrique est très spécialisée: elle est surtout caractérisée par sa propriété de ne pas colorer les graisses et les lipides et de, dans la classe des protéiques, ne colorer que certains types de substances.

Nous avons d'un autre côté montré que la réaction tanno-férrique peut servir de réaction matrice à plusieurs méthodes, que nous avons divisées en deux groupes: combinaisons prè-tanniques et post-tanniques.

Dans les prémières le tannin actue comme différentiateur après le colorant et comme mordant pour le fer; dans les deuxièmes, le

système tanno-férrique actue comme mordant complexe. Nous avons alors décrit comme principal combinaison pre-tannique la méthode de la safranine-tannin-fer, et comme types de combinaison post-tannique l'hématoxyline tanno-férrique et le tannin-fer-Giemsa (19).

À l'aide de ces techniques nous avons fait l'étude de plusieurs questions, en particulier de la zone de Golgi et de l'appareil para-Golgi, découvert en 1924 (4) avec la technique du tannin-fer simple. Nous avons également à l'aide du tannin-fer-Giemsa établi plusieurs faits nouveaux en hématologie, tels que la réaction cyanotannophile, les "granula tannophila", et d'autres faits qui nous ont permis de donner une autre classification des éléments du sang (pachy-neutrophiles, meta-neutrophiles, lepto-neutrophiles), en rapport avec l'évolution des éléments. Ces mêmes faits nous ont permis d'établir une différentiation plus nette entre les familles lymphoïde et granulocytique (20).

Ils nous ont permis de compléter le tableau cytologique des granulocytes, en bases nouvelles, de revoir, à partir de là, les idées d'Arneth et d'autres auteurs sur les formules neutrocytaires, l'équilibre neutrocytaire, etc. Ces mêmes faits nous ont permis encore de revoir certaines questions fondamentales de l'hématologie comparée (5).

La réaction du tannin-fer simple nous a permis encore en collaboration avec Ad. Estrada, de découvrir l'énigmatique réaction-choc des globules rouges et de l'hémoglobine (15).

La réaction tanno-férrique nous à conduit au concept de "tannophilie", "de substances tannophiles", accepté ensuite par les auteurs (Wallraff, Hruby, Stosiek, Tonutti, C. da Costa, Tavares de Sousa etc.), analogue à ceux de sidérophilie, argentophilie, etc., concept dont l'utilization a donné déjà des résultats positifs.

De la technique du tannin-fer dérivent encore les réactions du tannin-fer-acéto-ammoniacale et tannin-fer-transfert, introduites également par nous dans la cytologie et qui sont des réactions spécifiques du noyau (21).

La technique du tannin-fer et les méthodes qui en dérivent est caractérisée, d'après Beckert, "par sa simplicité, sûreté, vitesse et netteté de coloration" (22).

Ces méthodes ont determiné, d'après Wallraff (23), un certain renouvellement dans l'étude de plusieures questions cytologiques de l'hypophyse, de la thyroide, de l'estomac, du pancréas, etc. (23–28).

Ils ont permis également une analyse plus poussée de la zone et du complexus de Golgi, en particulier la découverte de l'appareil para-Golgi, le composant protéique du complexus (29).

Sous le point de vue de la coloration du conjonctif, d'après Tonutti, "die Méthode eignet sich im übrigen ausgezeichnet zur Wiedergabe der Präparate im Lichtbild und was mir schliesslich als das Allerwichtigeste erscheint, die Darstellung der Bindegewebsubstanzen ist unabhängig von Differenzierungseinflüssen, und damit objektiv. Ihre Anwendung bei vergleichenden Untersuchungen ist daher besonders zu empfehlen" (28).

L'analyse du conjonctif à l'aide des techniques au tannin-fer n'a pas encore été faite; du reste il y a encore un vaste champ de recherches à réaliser avec l'application de ces méthodes au tissu nerveux et dans plusieurs organes.

Nous avons montré déjà que ces méthodes peuvent être utilisées en cytologie végétale, et nous avons déjà montré, à l'aide de ces moyens, le non fondé de la théorie du vacuome (30) et de certaines théories sur les rapports entre le plastidome et le chondriome (32).

Tout récemment nous avons encore essayé, avec des resultats positifs, d'autres techniques au tannin-fer, conduisant à des resultats nouveaux.

Ces techniques, encore non publiées, ont pour base un mordançage tannique alterné et successif, jusqu'à obtenir une réaction noire intégrale suivie ou non de différentiation. Cette technique, que nous appellons tannin-fer-II, est un nouveau moyen d'analyse important, comme nous le montrerons sous peu.

Nous avons également essayé avec des résultats positifs, des combinaisons pre- et post-tanniques accouplées, à l'aide desquels ont peut réaliser de nouvelles méthodes polychromiques.

En resumé, la technique du tannin-fer, a permis déjà d'établir un ensemble considérable de faits, concepts et théories nouvelles en histologie et cytologie. Elle a été l'origine, d'un autre côté, de toute une série, déjà assez nombreuse, de nouvelles méthodes histologiques et ctyoloqigues, telles que la méthode de Wallraff, le tannin-fer-Giemsa, l'hématoxyline tanno-férrique, la safranine-tannin-fer et plusieures autres. On peut dire que la réaction du tannin-fer a créé déjà un chapitre nouveau de la technique histologique, chapitre qui n'est pas encore clos et dont les ressources sont encore en grande partie à explorer (22, 23, 24, 25, 26, 27).

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THE RAPID PREPARATION OF CELLOIDIN SERIAL SECTIONS FOLLOWING INDIA INK INJECTIONS

JAMES F. FEENEY JR., *University of California, Berkeley, Cal.*

ABSTRACT.—**For the study of capillary penetration in the central nervous system of the chick embryo, following India ink injections, celloidin serial sections are superior to those prepared by the paraffin technic. The celloidin sections are arranged on a moist cigarette paper mat, which when filled is inverted and applied to a microscope slide so that the sections contact the glass surface. Subsequent to dehydration and clearing the sections are isolated on the slide by peeling off the cigarette paper. Forty-five minutes are required to prepare a slide of thirty sections from the time the block is trimmed until the cover slip is mounted with Clarite.**

If the details of capillary penetration into such structures as the central nervous system are to be studied following India ink injections of chick embryos, serial sections are needed. Paraffin serial sections are not adequate for this purpose since removal of the paraffin allows the carbon particles to escape from the capillaries. Celloidin sections are essential for restricting the carbon particles to the lumen of the blood vessels. However, the usual methods for preparing celloidin serial sections are much too tedious and time-consuming to be useful. Consequently a method for the rapid preparation of celloidin serial sections has been developed and is described below.

The embryos should be fixed in Zenker's formol as described by Guyer (1936) since such fixation may be followed by a wide variety of stains. After fixation and thorough washing in tap water, the embryos are stained *in toto* with 1.0% alum carmine for 24 hours. (Other useful stains include tincture of cochineal with $CaCl_2$ and and $AlCl_3$, iron cochineal, magnesium carmine, and hydrochloric carmine; see Lee, 1937, pp. 139–49, for details.) They are then rinsed in tap water, and are next stained for 24 hours in 0.5% orange G. The orange G has a solubility of 1.80% in water and .22% in alcohol which permits differentiation of the alum carmine during subsequent dehydration in alcohol without removal of all of the cytoplasmic stain. A quick celloidin infiltration, such as the one described by Guyer (1936) lends itself well to the preparation of specimens up to 96 hours of incubation. Older specimens require longer periods for complete infiltration. The knife holder is clamped to a seven-inch microtome blade at its distal end with reference to the

operator. Cigarette paper, cut the size of a microscope slide, serves as a transferring mat. It is placed on the end of the blade opposite the knife holder (Fig. 1). During the sectioning process the microtome blade is flooded with an excess of 80% alcohol containing some orange G. This stain must be present in the alcohol solution in order to prevent the complete diffusion of the orange G from the sections. Only enough of the stain is needed to give the alcohol an amber color; otherwise unsightly crystals will form on the sections. The meniscus of alcohol formed along the cutting edge of the microtome knife should be in contact with the mat. Any extreme excess of alcohol will spill off the blade at the end of the stroke so that it is almost impossible to use too much of this solution. As the sections are cut they are guided down the knife and onto the

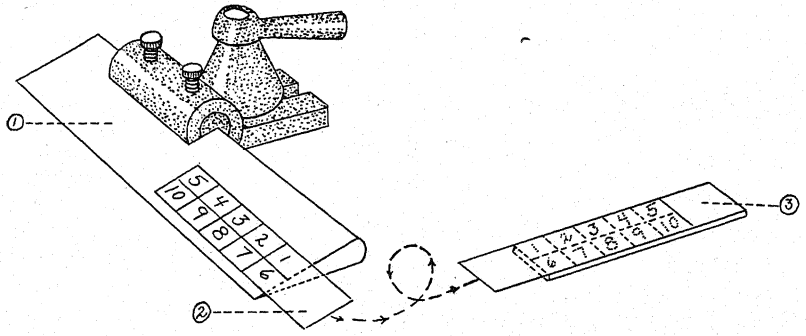

FIG. 1.

(1) Microtome blade. (2) Cigarette paper mat. (3) Slide.

cigarette paper with a No. 1 camel's-hair brush. Movement of the sections on the mat is made possible by directing the flow of alcohol, held in a No. 1 camel's-hair brush, under the section followed by gentle strokes for proper orientation. The sections are arranged in order on the paper from right to left (Fig. 1). Capillary action draws enough alcohol to the surface of the cigarette paper to keep the sections moist without allowing them to move from their position. The mat is raised higher on the blade when the last row of sections is being arranged to prevent any shifting of the sections due to movements of the alcohol meniscus. When the mat is filled with sections it is gently lifted from the blade with the left hand, inverted, and then applied to the slide (Fig. 1). When sectioning is discontinued the celloidin block should be covered with a cotton swab saturated in alcoholic orange G solution. While the mat is being lowered onto the

slide it should be guided with a pair of forceps held in the right hand. The margins of the cigarette paper and slide will accurately coincide if this procedure is followed. Care must also be taken to prevent air bubbles from forming between the glass surface and the sections. After the paper is oriented with respect to the slide there should be enough room on the right hand margin for the label. A blotter is then carefully pressed to the mat so that no sections are forced out from under the cigarette paper.

Dehydration is accomplished by applying 95% alcohol to the exposed surface of the mat, followed by blotting after sufficient time has elapsed for complete penetration. Three such rinses with alcohol followed by blotting are necessary to remove the excess orange G that has become included within the celloidin. Carbol-xylene is then applied to the mat and allowed to remain for 3 minutes. This

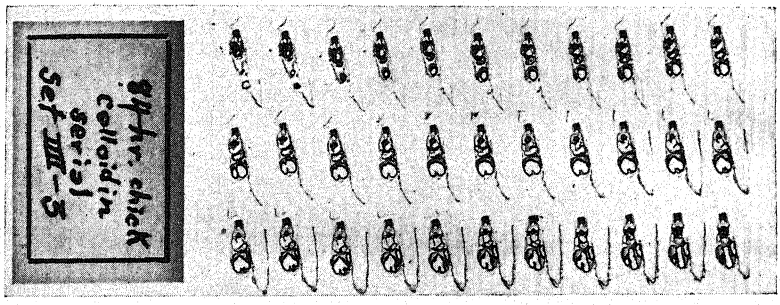

FIG. 2. Sample slide. × 1½.

period is usually sufficient to clear the sections; however, a longer application may be necessary on thicker specimens. After thorough blotting, the sections are left adhering to the slide by peeling off the cigarette paper. If the blotting has been complete there is no tendency for the sections to peel off with the mat. Thin Clarite is then added to the sections before they dry. Cover slips are applied, and are pressed tightly to the slides.

Once the block has been trimmed, a slide containing 30 serial sections, complete with cover slip, can be prepared in 45 minutes (Fig. 2). Although this procedure was developed for chick embryos, it can be readily applied to any animal or plant tissue. Moreover, if the staining is not done *in toto*, the sections can be covered with a thin film of celloidin after dehydration and stained like paraffin sections.

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MODIFICATION OF PROCEDURE FOR DIFFERENTIATING THE TELIA OF *CRONARTIUM RIBICOLA* AND *C. OCCIDENTALE*

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Telia of the pine rusts, *Cronartium ribicola* A. Fisch. and *C. occidentale* Hedgc., Bethel, and Hunt, on the leaves of *Ribes* are morphologically indistinguishable. The ranges of these rusts now overlap in parts of the West and, since one is under quarantine regulation and the object of a large-scale control program while the other is of little consequence, a method of reliable differentiation is required. Acree and Goss[1] have worked out a microchemical method that has proven successful in distinguishing between the two species at the telial stage, but if large numbers of tests are being made a shorter schedule than that described by them is advantageous. Accordingly, attempts to work out a shorter schedule were made. The following procedure was found to give as satisfactory differentiation between the species of rust as the longer method when both schedules were compared on more than 184 specimens over a period of three seasons:

1. Under a dissecting microscope cut out a piece of *Ribes* leaf blade approximately 5 × 5 mm. on which grow mature ungerminated telia. Germinated or parasitized telia do not give a satisfactory reaction.

2. Place in watch glass and cover with 2 ml. N/10 HCl. Under the dissecting microscope tease out all air bubbles with a camel's hair brush so that all parts of the telia are in contact with the acid. Leave specimen, telium-side down, in acid 5 minutes.

3. Remove specimen, pass through distilled rinse water and wash by immersion for 10 minutes in distilled water, making two changes.

4. Remove specimen and blot on filter or neutral blotting paper. Place on slide under dissecting microscope and tease telia from leaf blade with a dull scalpel.

5. Allow telia to dry (1–3 minutes) before putting on drop of .001 *M* brom phenol blue with pH adjusted to 7.6. Place cover glass in

[1]Acree, Ruby J., and Goss, Warren H. 1937. A microchemical colorimetric pH procedure for differentiating the telia of *Cronartium ribicola* and *C. occidentale*. J. Agr. Res., 55, 347–52.

position and set aside 20 minutes before examining under low power of comparison or compound microscope.

Spore contents of *Cronartium ribicola* telia turn blue and those of *C. occidentale* green. If the color is not clear because of the large amount of brom phenol blue under the cover glass, place a drop of distilled water at one edge of the cover glass and with filter paper draw off the indicator from the other side.

It should be emphasized in examining the telia that the color to be observed is that of the spore contents and not that of the surrounding matrix. The latter is usually yellow and because of this *C. ribicola* telia sometimes appear greenish blue as a whole. Moreover, the bases of telia of *C. occidentale* are sometimes blue. This may be due to a failure during the acid bath to eliminate air bubbles around the telia where they arise from the leaf blade, so that the acid does not come in contact with them. Occasionally telia are encountered which stain only in the outer layers. Acree and Goss[2] have described the typical colors obtained with their schedule as being those found in Maerz and Paul[3], plate 34 L 7–12 for *C. ribicola* and for *C. occidentale* plate 30 L 6–12. In Ridgway[4], for *C. ribicola* they are plate VIII, light cerulean blue, cerulean blue, oxide blue, and Antwerp blue, and for *C. occidentale* plate VII light blue-green, Guinea green, and dark viridian green, and plate XVIII oriental and dark yellowish green. With the shortened schedule, shades of blue and green similar to, or approximating these colors, are obtained.

Where a number of specimens are to be examined time may be saved by running up to four samples concurrently, using the requisite number of additional watch glasses and wash bottles or beakers. In the intervals during the processing of one group of samples, others may be prepared for treatment.

[2]See footnote 1.

[3]Maerz, A., and Paul, M. R. 1930. A dictionary of color. 207 pp. Illus. New York.

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NOTES ON TECHNIC

On the Cutting of Tough and Hard Tissues Embedded in Paraffin.

It has been known for some years that tissue embedded in paraffin has the power of taking up water, and that it is possible, after immersion in water of the tissue exposed by cutting into the paraffin block, to obtain a section from an otherwise uncuttable tissue. With animal tissues, however, this has certain disadvantages, and so Baker[1] was led to experiment with various other soaking fluids. The mixture of 9 parts 60% ethanol with 1 part glycerin that he found most satisfactory depends for its helpful action on the glycerin; the water and ethanol apparently act merely as accelerators of penetration. The main use of this fluid is stated by Baker to lie in the easier and finer cutting of brittle tissues such as liver, spleen or tissues containing much blood clot, a statement which our departmental experience very fully confirms. He then warns the reader that this mixture does not facilitate the cutting of tough tissues, of which he mentions as an example the formalin-fixed vagina of a bitch, but experience with the biopsy material in this hospital has shown that Baker's disclaimer is unduly cautious[2]. Tissues such as thyroid or scirrhous carcinoma of breast may well prove tough enough; and in friendly disagreement with Baker we have found that, if it prove difficult to obtain a decent section of such tissues, immersion of the block overnight in his mixture (or the similar mixture, marketed as Mollifex by the British Drug houses Ltd.) allows the cutting next morning of a thinner and better section. The value of this manoeuver is attested both by those who cut the sections and by those who must make the diagnoses.

More recently I have found that tough and hard human tissue is made even more amenable by soaking the opened paraffin block in a mixture of 9 parts of glycerin with 1 part of anilin. To achieve the best result may demand two or three days' soaking; this discolors the wax but seems to produce no other detrimental effect.

Thus to sum up our routine and the place of these adjuvants, paraffin blocks are mounted on hard wooden chucks and cut by the Cambridge rocking microtone. If the technician is dissatisfied with

[1]Baker, J. R. 1941. A fluid for softening tissues embedded in paraffin wax. J. Roy. Micr. Soc., **61**, 75–8.

[2]Lendrum, A. C. 1943. On the handling of small biopsy material. Brit. Med. J., **1943 ii**, 644.

the section obtained, he transfers the block, still attached to the wooden chuck, to a dish containing a thin layer of Baker's fluid. The block lies open face down in the fluid till the following morning; it is then usually possible for him to produce the promised thinner and better section. If, however, the tissue be especially hard or if a section be wanted of demonstration quality from such difficult tissues, the block is soaked for two or three days in the anilin-glycerin mixture. ALAN C. LENDRUM, *Department of Pathology, The University and Western Infirmary, Glasgow, Scotland.*

STANDARDIZED TABLETS FOR THE RESAZURIN TEST.

While there has been a steadily increasing interest in the resazurin test for reduction in milk, one drawback to its widespread use among the smaller dairy plants has been the necessity for weighing out accurately small quantities of the dye powder in preparing a solution of the desired strength. This drawback has now been removed. A batch of tablets of resazurin, each of which will make up 200 ml. of working solution when dissolved in that amount of water, has been prepared by the National Aniline Division of the Allied Chemical and Dye Corporation. Samples of these submitted for testing by the Biological Stain Commission have been found quite satisfactory and this batch has been certified by the Commission. These tablets should now be available through the regular laboratory supply houses.—C. K. JOHNS, *Central Experimental Farm, Ottawa, Canada.*

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

DYES AND THEIR BIOLOGICAL USES

COPLEY, A. L., and WHITNEY, D. V. **The standardization and assay of heparin by the toluidine blue and azure A reactions. A correction.** *J. Lab. & Clin. Med.*, 29, 117.

An error occurred in the previous paper (*J. Lab. & Clin. Med.*, 28, 762. 1943 Stain Techn. 19, 48). Throughout the paper, all the concentrations of toluidine blue and azure A, and all quantities given as gamma should be read one tenth of the reported values.—*John T. Myers.*

FINKELSTEIN, JACOB. **N^4-substituted sulfonamides.** *J. Amer. Chem. Soc.*, 66, 407. 1944.

A series of chloracetyl, aminoacetyl and caproyl derivatives of sulfonamides was prepared and tested for their efficacy as chemotherapeutic agents. Experiments in mice suggested that these compounds have a low acute toxicity similar to sulfadiazine, sulfapyridine and sulfathiazole, and showed that many of the derivatives are very effective against such organisms as *Streptococcus*, pneumococcus (Type I), *Staphylococcus aureus* and *Salmonella schottmülleri*. The relative activity of these drugs was also tested against *Plasmodium lophurae* in Pekin ducklings. The caproyl compounds all showed about the same activity as the parent sulfonamides and the chloracetyl derivatives seemed uniformly less active.—*A. P. Bradshaw.*

FRAENKEL-CONRAT, H., and COOPER, M. **The use of dyes for the determination of acid and basic groups in proteins.** *J. Biol. Chem.*, 154, 239. 1944.

The authors recommend the following reagents for the determination of the acid and basic groups in proteins. Dye solutions: 0.1% aqueous orange G (Coleman and Bell) and 0.2% safranin O (National Aniline); these percentages were computed from material dried to constant weight at 70° C, disregarding the "dye content" indicated on label. Buffers: for pH 2.2, 980 ml. 0.1 *M* citric acid and 20 ml. 0.2 *M* Na_2HPO_4; for pH 11.5, 250 ml. 0.2 *M* Na_2HPO_4 and 200 ml. 0.1 *N* NaOH, water to make 1000 ml.

The method recommended is: To each of four 15 ml. test-tubes suitable for use in angle head centrifuge are added 5 mg. of the protein, 1 ml. of the pH 2.2 buffer, increasing amounts (1, 2, 3 or 4 ml.) of 0.1% orange G and two glass beads. If the protein dissolves in the buffer, it will be precipitated by the dye. The suspensions are shaken mechanically for 20–24 hours. They are centrifuged and aliquots of supernatant solution are diluted 100-fold. Color intensities are determined by means of a photoelectric colorimeter (Klett-Summerson) with a blue filter (Corning No. 038 amd Pyrex No. 554). Dye concentrations are read from a standard curve prepared from data obtained with appropriate dilutions of orange G stock solution. The dye bound by the protein is determined by subtracting the excess found in the supernatant fluid from the amount added. The same method is used with the alkaline buffer and the safranin solution. Details of the results obtained by this method using various proteins are given in tabular form and are discussed.—*J. E. Kindred.*

GILMAN, HENRY, and SHIRLEY, DAVID A. **Some derivatives of phenothiazine.** *J. Amer Chem. Soc.*, 66, 888. 1944.

Because earlier studies have shown certain phenothiazine derivatives (e.g. methylene blue) to have antimalarial activity the authors prepared some appropriately substituted, non-oxidized phenothiazines for testing in avian malaria.

It has been shown that phenothiazine and some of its simple derivatives have a low toxicity for animals, and that this toxicity is further reduced by substitution in the 10- position. For this reason, some 10-phenylphenothiazine derivatives were prepared. The 10-(4′-γ-diethylaminopropylamino)-phenylphenothiazine was the only one of these compounds which showed any activity, and this was of a doubtful nature.—*A. P. Bradshaw.*

GILMAN, HENRY, and SPATZ, SYDNEY M. **Some quinolines patterned as "open models" of atabrine.** *J. Amer. Chem. Soc.*, **66**, 621. 1944.

In connection with studies attempted on the correlations of constitution with antimalarial action, four compounds having essentially the same functional groups as atabrine but having a more open molecule were prepared. Antimalarial activity in avian malaria was shown by both 6-methoxy-2-(3′-chlorophenyl)-4-[(α-methyl-δ-diethylaminobutyl)-amino]-quinoline and the isomeric (4′-chlorophenyl) compound, thus showing that the position of the chlorine in this part of the molecule did not affect the activity. The presence of the chlorine was also shown not to be essential for activity, for 6-methoxy-2-(phenyl)-4-[(α-methyl-δ-diethylaminobutyl)-amino]-quinoline is also active. However the position of the methoxy group (in the chlorine free type) is important, because 2-(2′-methoxyphenyl)-4-[(α-methyl-δ-diethylaminobutyl)-amino]-quinoline, unlike the 6-methoxy isomer, is inactive.—*A. P. Bradshaw.*

KLOTZ, IRVING M. **The mode of action of sulfonamides.** *J. Amer. Chem. Soc.*, **66**, 459. 1944.

The inhibition of bacterial growth by sulfonamides may be accounted for quantitatively by assuming that the action is due to a reversible combination between the basic form of the drug and the neutral form of the protein, and that the law of mass action is applicable. Equations may be derived which relate drug potency to the acid ionization constant of the sulfonamide and to the pH of the solution.

The reversal of the sulfonamide bacteriostasis by addition of *p*-aminobenzoic acid may be considered from the same point of view. Expressions may be obtained which account for variations in the ratio of sulfonamide to *p*-aminobenzoic acid from drug to drug and from one pH to another.—*Author's summary.*

MUELLER, ALBERT C. and HAMILTON, CLIFF S. **Some derivatives of 7-methoxy- and 10-methoxybenzoquinoline.** *J. Amer. Chem. Soc.*, **66**, 860. 1944.

The synthesis of two series of methoxybenzo(f)quinolines with a basic substituent in the 1- position has been undertaken with the hope that the presence of the methoxyl would increase the antimalarial activity of this type of compound. Pharmocological testing of a number of these derivatives is in progress.—*A. P. Bradshaw.*

PETERSON, OSLER L. **Therapeutic effects of forbisen and of toluidine blue on experimental typhus.** *Proc. Soc. Exp. Biol. & Med.*, **55**, 155–7. 1944.

Mice infected with yolk sac strains of typhus were protected by toluidine blue used in concentrations of 0.25 to 0.75% in the diet, or 5 to 20 mg. per day. Forbisen even at a level of 2.5% was less efficacious. Infected cotton rats were not protected by these substances.—*M. S. Marshall.*

ANIMAL MICROTECHNIC

BALLANTYNE, E. N. **A staining method for frozen sections.** *Canad. J. Med. Techn.*, **2**, 65–7. 1940.

The following method is sufficiently rapid for quick diagnosis, and yields satisfactory permanent preparations: Fix tissue in 10% neutral formol, containing 0.85% NaCl, by heating solution to boiling. Wash in tap water. Place on freezing microtome in a drop of dextrin solution. Freeze, cut, and place sections in distilled water. Wash section in distilled water. Stain 10 sec. in 0.1% aqueous phloxine. Wash in distilled water. Stain 10 sec. in 0.5%

aqueous toluidine blue. Transfer to 50% ethyl alcohol, move about quickly to remove loose stain. Place in 80% alcohol for a few seconds. Float onto slide in distilled water. Drain off water and cover section with drop of 50% alcohol. Blot with clean white blotting paper. Drop 80% alcohol on the section, drain off excess. Drop 95% alcohol on section, drain off excess. Dry slide edges carefully. Cover section with drop of euparol mounting medium and apply cover slip.—*C. Randall.*

BODIAN, DAVID, and MELLORS, ROBERT C. **Phosphatase activity in chromatolytic nerve cells.** *Proc. Soc. Exp. Biol. & Med.*, 55, 243–5. 1944.

The authors found chromatolysis to be indicated by degradation of chromophilic material, a nucleoprotein of the ribose type, found in nerve cells. Increase in acid phosphatase activity was proportional to the degree of chromatolysis. Phosphatase activity was demonstrated as areas in sections darkened due to lead sulfide. These sections were prepared from spinal cord, soaked in a phosphate ester and lead nitrate. Inorganic phosphate, released by enzyme activity, was precipitated by lead nitrate, and lead sulfide was formed by treatment with ammonium sulfide.—*M. S. Marshall.*

FORBES, J. **Glycerine jelly mounting medium for frog eggs and early embryos.** *Trans. Amer. Micr. Soc.*, 62, 325–6. 1943.

Frog eggs or embryos in alcohol, water, or formalin may be mounted directly in glycerin jelly for use by students. The jelly is prepared as follows: soak 28 g. of gelatin in 120 ml. distilled water until soft; add 140 ml. glycerin; heat gently until gelatin is dissolved; add 2 ml. melted phenol; filter twice through glass wool in a heated funnel; collect the second filtrate in an agate-ware pie pan; brush bubbles to one side; allow to solidify; cut into 1 inch squares; and wrap in No. 72 waxed powder paper. To use for mounting, heat a square very gently on the slide by superimposing it on another slide which has been heated, then put the egg in place and cover it.— *Virgene Kavanagh.*

HUGHES, R. F. **Laboratory hints.** *Canad. J. Med. Techn.*, 3, 25–6. 1940.

The following technic is excellent for differential staining of eosinophilic granules in nasal discharges: Tease out secretion on slide and dry gently over flame. Stain with 0.5% alcoholic eosin, 1 min. Add enough distilled water to take up staining solution and let stand 1 min. Drain off and wash with distilled water. Flood with 95% ethyl alcohol. Drain off the alcohol, and immediately stain 1 min. with 0.1% alcoholic methylene blue. Flood slide with distilled water and let stand 2 min. Remove excess stain with distilled water. Cover slide with ethyl alcohol. Drain off the alcohol, and examine. Restain with methylene blue if neutrophiles do not stain well. If blue is too intense, flood slide with weak acid solution (1 drop 1% HCl in 1 ounce distilled water); quickly pour off, flood slide with distilled water, and finally with ethyl alcohol.—*C. Randall.*

KRAJIAN, ARAM A. **Elastic fibre stains.** *Canad. J. Med. Techn.*, 3, 207. 1941.

The author proposes the following technic: Fix tissues in 10% formaldehyde 24 hr. or longer. Cut frozen sections about 10 μ. Place in 2% aqueous $AlCl_3$ for 5 min. Wash thoroughly in tap water. Place for 10 min. in the following stain: 4% aqueous Congo red, 8 ml.; glycerin (C.P.), 2 ml. Wash quickly in tap water. Rotate section for 10 sec. in 1% aqueous KI. Wash thoroughly in tap water. Place for 5–10 min. in the following stain: resorcinol, 3 g.; aniline blue, 1.5 g.; orange G, 2.5 g.; phosphomolybdic acid, 1.0 g.; distilled water, 100 ml. Wash in tap water. Float section onto slide and blot with filter paper. Dehydrate 2 min. in 3 changes of absolute alcohol (from dropping bottle). Clear for 2 min. in oil origanum. Clear in pure xylene. Mount in gum dammar.

This method gives a well-differentiated preparation in which elastic fibers appear bright red; fibrin and connective tissue, dark blue; red blood cells, orange yellow.—*C. Randall.*

KUPPERMAN, H. S., and NOBACK, C. R. **A rapid iron hematoxylin tissue stain for laboratory use.** *Science*, 98, 591–2. 1943.

A modification of the standard Heidenhain iron hematoxylin stain is described in which the processes of fixation and mordanting are executed simultaneously in a solution prepared by using 1.5 g. ferric ammonium sulphate per 100 ml. of Bouin's fluid, the time for fixation and mordanting for small (5 to 6 mm. cube) pieces of tissue being the same as usual. After removal of the tissue from the fixative-mordant, it is stained with 0.5% hematoxylin, prepared for mounting by the usual procedure, using either alcohol or dioxane. When sections are passed through alcohol to the stain, the process must be rapid to avoid washing out the mordant. Sections are overstained in 3 to 5 minutes and destaining may be carried out as desired in 0.1% aqueous or 35% alcoholic HCl, followed by an alkaline wash in a solution prepared by adding several ml. of saturated aqueous $LiCO_3$ or 1% $NaHCO_3$ to 100 ml. of either water or 35% alcohol.

Mordanting just prior to the staining is unnecessary. This modification of the usual procedure makes a rapid and simple stain for routine laboratory use with the resulting stain comparable to that observed after use of the accepted and lengthy Heidenhain iron hematoxylin method.—*T. M. McMillion.*

LAWS, S. G. **A method of staining blood films.** *Canad. J. Med. Techn.*, 3, 63. 1941.

The following method for staining occasional blood films employs stock solutions of 1% aqueous azure II and 1% aqueous eosin that may be kept indefinitely: Fix blood films. Mix 1 ml. of diluted Azure II (stock solution diluted 10 times) with 9 ml. dilute eosin (3 ml. stock solution plus 500 ml. distilled water). Pour a little on slide and let stand 20–30 min. Wash in distilled water and examine.—*C. Randall.*

LILLIE, R. D. **Studies on the decalcification of bone.** *Amer. J. Path.*, 20, 291–6. 1944.

A variety of decalcifying agents were experimentally tested for their ability to decalcify bone and still permit staining of the blood elements with Romanovsky stains. It was found that satisfactory Romanovsky stains could be secured in decorticated cancellous bone decalcified with NH_4NO_3, NH_4Cl or KH_2PO_4, all of which had an initial pH of 5.0 or slightly above. Similar good results could be obtained on corticate bone by decalcifying with mixtures of sodium citrate and formic acid buffered to an initial pH above 3.0. Mixtures of formic acid and sodium citrate with a pH below 3.0 did not give as good results; these were buffered with mixtures of citric acid and K_2HPO_4.—*J. O. Foley.*

MOORE, MARGARET E. **Twenty-hour schedule for routine tissue sections.** *Canad. J. Med. Techn.*, 2, 70–1. 1940.

To speed up results where 24-hour laboratory service is available, the author recommends the technic here given. Keep tissues from 3 P.M. until 8 P.M. in modified Dubascq-Brasie fixative prepared as follows: formaldehyde, 80 ml,; acetic acid, 40 ml.; normal saline, 250 ml.; alcohol (used), 400 ml.; picric acid, 4 g. Run through four changes of methanol in 7 hr., 1 hr. in acetone, 2 hr. in each of two changes of toluene, 3 hr. in each of two baths of paraffin, and embed. Cut sections, adhere to slide with 1% aqueous gelatin, and dry in oven. Treat with xylene, alcohol, and water. Stain 5 min. with Mayer's acid hemalum prepared as follows: distilled water, 1000 ml.; hematoxylin, 1.5 g.; $NaIO_3$, 0.2 g.; potassium alum, 50 g.; chloral hydrate, 50 g.; citric acid, 1 g.; mix and let dissolve at room temperature. Place in running water until blue. Counterstain with 0.1% aqueous eosin 3–4 min. Wash in tap water, dehydrate in alcohol, clear in carbol xylene and xylene, mount in Canada balsam.—*C. Randall.*

PAUL, PAULINE, LOWE, B., and McCLURG, B. R. **Changes in histological structure and palatability of beef during storage.** *Food Research*, 9, 221–33. 1944.

The histological changes during the onset and dissolution of rigor and subsequent ripening of beef were investigated by the following technic: Slides were

made of both the raw and the cooked meat. The tissues were fixed in 10% formalin. Both paraffin and frozen sections were prepared. The paraffin sections were stained with French's modification of Weigert's elastic tissue stain, Harris' alum hematoxylin and van Gieson's mixture of acid fuchsin and picric acid. From the paraffin treatment the muscle fibers were stained yellow-orange, the collagenous tissue bright red, and the elastic tissue dark blue-green. The frozen sections were stained with Harris' alum hematoxylin and Herxheimer's "scarlet R" solution, giving blue muscle tissue and red fat deposits.

Rigor was shown histologically by the formation of dense nodes of contracture bordered on either side in the same fiber by areas of extreme stretch. Fibers which did not contract were drawn into waves and kinks by the shortening produced by the contracted fibers. Normal rigor produced denser nodes than those caused by heat when cooking of the beef was started before the onset of rigor.

The passing of rigor and progress of ripening were indicated by the appearance of breaks in the fibers. Sharp fractures usually occurred in the passively retracted fibers and granular or disintegrated areas in the stretched portions of the fibers adjacent to the rigor nodes.

The tests described were made on only one animal. Twenty pictures are given including a number of photomicrographs.—*William G. Walter.*

PINKUS, HERMANN. **Acid orcein Giemsa stain (modification of Unna-Taenzer method).** A useful routine stain for dermatologic sections. *Arch. Dermat. & Syph.*, 49, 355–6. 1944.

For dermatological staining the author combines acid orcein (1 g. orcein in 100 ml. 70% ethyl alcohol, with 0.6 ml. concentrated HCl; usable immediately, but improving on standing) with dilute Giemsa solution (1 drop of "any good Giemsa stock solution" to 20 ml. distilled water). All the staining is done in jars, and the procedure is as follows: Deparaffinize the sections in xylene; transfer to 95% alcohol; 70% alcohol; stain 30–60 min. with acid orcein solution; rinse 2 min., or more in distilled water; wipe off excess water and dip briefly into 95% alcohol; decolorize 5–30 min. in absolute alcohol. (The sections should now be pale brown; the elastic fibers should stand out deep purple or black when observed under low power.) Decolorize in acid absolute alcohol (one drop conc. HCl to 10 ml.) until the background is almost colorless. (This step increases contrast considerably, but it should not be extended over 2–10 min. lest thin elastic fibers become decolorized.) Transfer to tap water for 5 min., or longer. Stain 2–12 hr. with dilute Giemsa solution. (Epithelial and other cells now should be deep blue and connective tissue grayish pink, grayish blue or fairly deep blue.) Wipe off excess fluid and dehydrate in 95% alcohol containing a few drops of 1% alcoholic eosin Y, e.g. the alcohol used for dehydration of hematoxylin and eosin sections. (The tissues lose their blue tinge fairly rapidly in this step, and decolorization must be stopped when the connective tissue has just lost all trace of blue and has become faintly rose. The epidermis should remain bright blue.) Cover with absolute alcohol, 2 changes, for 2 min. each; xylene, 2 changes; mount in Canada balsam.

A well stained section shows the following contrasts: Nuclei, deep blue; the cytoplasm of the epidermis, muscle cells and connective tissue cells, light blue; plasma cells, dark grayish blue; eosinophilic granules, bright red; mast cell granules, metachromatic purple; neutrophilic granules, usually not well stained; red corpuscles, brownish red; collagenous fibers, light pink, possibly with a faint brownish tinge; elastic fibers, dark brown or black; senile degenerated connective tissue (collacin, elacin and collastin), presenting various combinations of black, gray and blue; cartilage, matachromatic purple; decalcified bone, light brown; keratohyalin, blue (usually not prominently stained); stratum lucidum, dark rose; the keratin layer, colorless, light blue or light pink, depending on the tissue reaction and the degree of decolorization; the inner root sheath of the hair, deep blue; melanin granules, green or greenish black; other pigment, particularly hemosiderin, unstained; bacteria and mycelia, deep blue; *Demodex folliculorum*, in hair follicles, brown with blue granulations.—*J. A. Kennedy.*

PUGSLEY, MARION L. **Reticulocyte staining.** *Canad. J. Med. Techn.*, 3, 16–7. 1940.

The following procedure is recommended: Prepare aqueous stain by dissolving 1 g. brilliant cresyl blue in 100 ml. 0.2% potassium oxalate. Mix one drop of stain with one drop of blood in small wax block. Blend by gentle blowing; allow to stand 1 min. Make smears and stain with any of the Romanovsky stains. Counterstaining with Wright's or Leishman's facilitates the examination of the smears, but reticulocytes may be counted with no further staining.

This method has three great advantages: the reticulum is heavier and thus more easily identified, the cells are not distorted, and the use of a centrifuge is obviated.—*C. Randall.*

SLAVKIN, ALICE E. **Quick paraffin method for small biopsies.** *J. Lab. and Clin. Med.*, 29, 74. 1944.

The following method is useful in the microscopic examination of fragments of tissue too small for frozen sections: Fix in 4% formalin or Zenker's fluid for 10 min. at room temperature. Stain in any hematoxylin for 1 min. Wash in tap water. Dehydrate in 80%, 95% and absolute alcohols each 3 min. Clear in xylene 5 min. Transfer to melted paraffin, 2 changes for 5 min. each at 56° C. Embed in paraffin. Cut thin sections, transfer to slides and dry for 5 to 10 min. at 50° C., cool and stain as usual. Sections may be obtained within an hour.—*John T. Myers.*

PLANT MICROTECHNIC

STUART, NEIL W., and EMSWELLER, S. L. **Use of enzymes to improve cytological techniques.** *Science*, 98, 569–70. 1943.

This is a preliminary note on the use of enzymes, particularly Clarase, a proprietary enzyme complex, to overcome clumping of chromosomes in the first meiotic metaphase of *Lilium*. Buds were fixed in a solution composed of three parts absolute alcohol to one part glacial acetic acid, then run through 95% alcohol to 70% alcohol for storage. For enzyme treatment the fixed buds were run through lower strengths of alcohol to water and thoroughly washed, then treated with the enzyme solution. With Clarase, treatment of 15 to 20 min. was as effective as several hours; definite reduction of chromosome clumping was obtained. Treatment of buds with several other types of solutions did not yield positive results.—*T. M. McMillion.*

WITTLAKE, EUGENE B. **Permanent prestaining in botanical microtechnic.** *Ohio J. of Sci.*, 44, 36–8. 1944.

The author presents a method of staining blue-grass leaves in which both the primary stain and the counterstain are up to standard as to brilliance and intensity. The procedure is as follows:

Cut leaves transversely into one-eighth inch lengths. Place material in formal-acetic-alcohol (70% ethyl alcohol) or Bouin's fluid, and by means of a vacuum pump, apply a vaccum of 600 mm. mercury for 12 hr. Rinse quickly in distilled water. Transfer to 48% commercial HF for 120 hr. Remove and wash 1 hr. in running tap water. Transfer to Johansen's 50% tertiary butyl alcohol and pump 1 hr. at 600 mm. mercury. Stain 96–120 hr. in safranin Y made according to Johansen's procedure of preparing safranin O with methocellosolve. Dehydrate by Johansen's tertiary butyl alcohol method up to 100% at 2 hr. intervals. Transfer at 2 hr. intervals to 10, 25, 50, 70, 85, 95, 100% toluene series made up in tertiary butyl alcohol. Transfer to equal parts of xylene and toluene, and finally to 100% xylene at 2 hr. intervals. Place in embedding oven for 30 min. To infiltrate, add rubber paraffin to xylene at 3 hr. intervals and pour off one-half volume of mixture until odor of xylene is removed. Section. Remove paraffin with xylene. Agitate slide 45 sec. in equal parts of xylene and absolute alcohol, then in absolute alcohol for 45 sec. Counterstain 30–45 sec. in Johansen's fast green FCF. Agitate 1 min. in Johansen's fast green rinse (1 part xylene; 1 part clove oil; 1 part 100% ethyl alcohol). Clear 2 min. in synthetic methyl salicylate. Complete in 2 changes of xylene at 2 min. intervals. Mount in xylene, balsam, or clarite:—*C. Randall.*

MICROÖRGANISMS

ALEXANDER-JACKSON, ELEANOR. **A differential triple stain for demonstrating and studying non-acid-fast forms of the tubercle bacillus in sputum, tissue and body fluids.** *Science*, 99, 307–8. 1944.

The following procedure is a modification of the Ziehl-Neelsen technic for demonstrating non-acid-fast forms of the tubercle bacillus: prepare smears which are not too thick and stain 3 min. with carbol-fuchsin; decolorize 1 to 3 min. with acid alcohol (3% HCl) and wash thoroughly in running water; flood slides with a well ripened Loeffler's methylene blue; then add 6 to 8 drops of freshly prepared *N* NaOH, letting stand for not more than 1 min.; flood the slides one at a time with sodium hydrosulfite solution, prepared by adding a "pinch" of sodium hydrosulfite to 50 ml. of tap water, to decolorize all except red acid-fast and blue non-acid-fast tubercle bacilli; wash off quickly in running water and flood the slide with an aqueous solution of equal volumes of 1% acid green and 1% acid yellow; wash off the stain and blot dry at once. This triple method of staining should be a useful supplement to the usual Ziehl-Neelsen technic since it reveals a number of interesting non-acid-fast forms which ordinarily escape observation.—*T. M. McMillion.*

BARRITT, M. M. **An improved Pappenheim stain for gonococci.** *Brit. Med. J.*, 494, 4344. 1944.

Suggestions to overcome some of the criticisms of the author's procedure and the variability in the dyes are given together with a modified formula: methyl green, chloroform treated, 0.1–0.5%, 100 ml.; absolute methanol, 10 ml.; pyronin G, 0.5 g. or more; phenol, 1 g.; and glycerol, 20 ml.—*H. Leverne Williams.* (Courtesy *Biological Abstracts*).

BARTHOLOMEW, J. W., and UMBREIT, W. W. **Ribonucleic acid and the Gram stain.** *J. Bact.*, 47, 415. 1944.

Pure crystalline ribonuclease rendered Gram-positive organisms Gram-negative under conditions in which the bacteria remained Gram-positive in the absence of the enzyme.— *V. Kavanagh.*

BAUER, WILLIAM H. **Tooth buds and jaws in patients with congenital syphilis.** *Amer. J. Path.*, 20, 297–319. 1944.

The author reports superior results with an adaptation of the silver technic of Bertarelli and Volpino, a modified Levaditi method for staining *Treponema pallidum* in bone. The tissues are fixed in 2% formaldehyde or alcohol, decalcified in 5% HNO_3, blocked and placed in 5% $Na_2SO_4 \cdot 10\ H_2O$ for 2 days. Subsequently they are washed in tap water for 2 days and placed in a freshly prepared acidified alcoholic solution of $AgNO_3$ ($AgNO_3$ 1.5 g., distilled water 50 ml., 96% alcohol 50 ml., glacial acetic acid 4 to 5 drops). The blocks of tissue remain in the silver bath at 37° C., in the dark, for 18 days. The silver solution is changed when it becomes cloudy. Following impregnation with silver the tissues are washed for 1 day in frequent changes of distilled water. After washing, the blocks of tissue are reduced (tannic acid 3 g., gallic acid 5 g., sodium acetate 10 g., distilled water 350 ml.) in the dark, at room temperature, for 48 hours. The reducing solution is changed when it becomes cloudy. Following reduction, the tissues are washed thoroughly in distilled water, dehydrated and embedded in celloidin or paraffin. If paraffin is used, cedar oil is interposed between xylene and paraffin. The organisms stain black; the tissue yellow. The sectioned tissues may be subsequently counterstained with hematoxylin and eosin if desired.—*J. O. Foley.*

BEGG, A. M., FULTON, F., and VAN DEN ENDE, M. **Inclusion bodies in association with typhus rickettsiae.** *J. Path. & Bact.*, 56, 109–13. 1944.

Rickettsiae in mouse and rabbit lung are readily demonstrated by the following modified Macchiavello method: Make thin impression smears of the cut surface of infected lungs. Fix by heat. Just before use mix 9 parts of 0.5% aqueous basic fuchsin solution with 1 part of M/15 phosphate buffer at pH 7.6, and filter

onto the slide. After 5 min. wash off the buffered fuchsin solution with tap water, and differentiate for 30–90 sec. with M/50 citrate buffer at pH 3.0. Wash with tap water. Counterstain for 30 sec. with 1% aqueous methylene blue. The rickettsiae are red and the cells blue. The results are illustrated with color photomicrographs.—*S. H. Hutner.*

CHEREWICK, W. J. **Studies on the biology of Erysiphe graminis DC.** *Canad. J. Research*, 22, 52–85. 1944.

This is a study of the distribution and prevalence in Canada, the method of overwintering, the physiologic specialization, and the effects of various environmental factors on the development of the fungus *Erysiphe graminis.* To determine whether the conidia were uninucleate, they were dusted onto a slide lightly smeared with egg albumin. The slide was then flooded with aceto-carmine and a small crystal of hematoxylin was added; the slide was placed in a moist chamber at ordinary room temperature for 18 to 24 hr. The nuclei were well stained. Equally good results were obtained if the slide was warmed slightly for 4–6 hr.—*H. P. Riley.*

DISSMANN, EDWIN. **Erfahrungen mit der karbolnachtblaufärbung der Tuberkelbazillen nach Hallberg.** *Zentbl. Bakt., I Abt. Orig.*, 150, 268–75. 1943.

The author suggests the following technic as an improvement over the classic Ziehl-Neelsen method for staining tubercule bacilli. Cover air-dried and flamed smear with night blue solution prepared in the following manner: dissolve 5 g. night blue (Grübler) in 100 ml. 95% alcohol (*solution A*); add 0.2 ml. 10% KOH to 100 ml. distilled water and then add 2.5 ml. liquid phenol (*solution B*); combine 1 part solution A with 10 parts solution B immediately before use. Slowly warm to boiling, cool 5 min., pour off solution. Destain in a mixture of 3 ml. 25% HCl and 100 ml. 70% alcohol until all possible blue has been removed. Wash with distilled water. Counterstain 5–10 sec. with 2% aqueous Bismark brown. Wash with distilled water and dry. (As alternate counterstains, the author recommends an acetic neutral red, a carbol pyronin, or a diluted carbol fuchsin.)

It was found that this technic has both qualitative and quantitative advantages. The tubercule bacilli were more darkly stained. Granules, often hard to recognize, appeared dark blue against the fine, pale blue stroma. In addition, when similar smears were stained by the Ziehl-Neelsen technic and with night blue, the latter method gave a larger bacterial count.—*C. Randall.*

HUNT, GEORGE A. **A study of the Pappenheim stain. A stable modification.** *J. Lab. & Clin. Med.*, 29, 207–10. 1944.

The following formula is suggested: Mix 1.2 ml. of 1% aqueous malachite green (oxalate, certified "NMg 3", dye content 75%), 3.0 ml. of 0.5% aqueous pyronin G (certified "NP-7", dye content high) or pyronin 2 G (Coleman and Bell), and 20 ml. of distilled water. Prepare a stock solution of the malachite green 2 days before use and filter. Apply the stain for 30 sec. to 3 min. and wash with water. The stain is of special value in the examination of blood specimens, heavy smears, and blood clot cultures of chancroidal pus. Ducrey's bacillus stains much more distinctly than by the Gram technic.—*John T. Myers.*

KIRSH, DAVID, and SCHENKEN, JOHN R. **A comparison of the Ziehl-Neelsen and the Moss cold carbol fuchsin stains for acid-fast bacilli.** *New Orleans Med. and Surg. J.*, 96, 394–6. 1944.

In contrast to the conventional Ziehl-Neelsen stain for acid-fast bacilli, the Moss cold carbol fuchsin method consists in fixing the smears in commercial formalin for 8 minutes, rinsing in water, and then staining with cold carbol fuchsin for about 4 minutes at room temperature. The advantage of this method is the avoidance of steaming the slide and the resultant saving of time. The Moss cold carbol fuchsin method gave as satisfactory stains as the Ziehl-Neelsen method.—*Robert Schrek.* (Courtesy *Biological Abstracts*).

LEE, H. I. **Comparison of procedures for staining tubercle bacilli in fluorescent microscopy.** *J. Lab. & Clin. Med.*, 29, 218–21. 1944.

As compared with a number of alternatives, the following is the best procedure: Cover the smear for 5 min. with 0.1% auramine containing 5% phenol. Wash with tap water. Decolorize with 25% concentrated H_2SO_4 in 70% alcohol. Counterstain with 1:1000 aqueous $KMnO_4$ which overcomes interfering fluorescence. Staining with methylene blue is unnecessary.—*John T. Myers.*

NOBLE, GLENN A. **A five-minute method for staining fecal smears.** *Science*, 100, 37–8. 1944.

A rapid procedure, well adapted to staining protozoa in fecal smears, is proposed: With a brush make a very thin smear of the material to be examined; cover smear immediately with an iron alum solution [formalin 10% by vol., 3 parts; glacial acetic acid, 1 part; $FeNH_4(SO_4)_2 \cdot 12H_2O$, 3 g. per 100 ml. of formol-acid mix]; pass slide through a flame until the fixative begins to steam; add more solution to the slide to prevent drying and quickly pour off the mordant fixative; immediately add several drops of 0.5% aqueous hematoxylin and pass slide through flame one or two times, the fecal material becoming a dark purple in 3 or 4 seconds; wash in running tap water in a Coplin jar 1 min.; remove all excess water from the slide with blotting paper and transfer the slide to dioxane; put the slide in a second jar of dioxane, 1 min.; transfer to a solution of dioxane and toluene, half and half, 30 sec. or more; mount in clarite.—*T. M. McMillion.*

HISTOCHEMISTRY

DISCHE, ZACHARIAS. **Two characteristic and sensitive color reactions between sulfhydryl compounds and thymonucleic acid.** *Proc. Soc. Exp. Biol. & Med.*, 55, 217–8. 1944.

Thymonucleic acid and cysteine react with each other in H_2SO_4 at 40° with the formation of pink or reddish-brown end products, probably due to the formation of some furfuryl compound from desoxyribose. Cysteine in $1:10^6$ and thymonucleic acid in $1:10^4$ can be detected. Glutathione, 1:300,000, also reacts. The character of the reaction varies depending upon which of the two reactants is in excess.—*M. S. Marshall.*

WILMER, HARRY A. **Failure to demonstrate alkaline phosphatase activity in inclusion bodies by the histochemical technic.** *Proc. Soc. Exp. Biol. & Med.*, 55, 206–7. 1944.

Alkaline phosphatase activity was not demonstrated in inclusion bodies of vaccinia, herpes simplex, fowlpox, or tracheo-laryngitis. The technic, modified from Gomori's method, was based on staining for insoluble calcium phosphate precipitated at the site of enzymic activity. Alcohol-fixed sections were incubated in a solution of sodium-β-glycerophosphate and $CaCl_2$ at the proper pH.—*M. S. Marshall.*

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